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VOLUME 37, NO. 3 SEPTEMBER 1960

THE INCORPORATION OF ^{14}C FROM SODIUM ACETATE-2-^{14}C INTO THE AMINO ACIDS OF THE SOIL-INHABITING NEMATODE, *CAENORHABDITIS BRIGGSAE*

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(With Plate 8)

INTRODUCTION

The experiments described in this paper were intended to throw light on the nutrition of nematodes. The soil-inhabiting nematode, *Caenorhabditis briggsae*, which has already been the subject of much nutritional work, was chosen for the experiments. It has the advantage that it can be cultured indefinitely under axenic conditions, thereby avoiding the complications introduced into much nutritional work by the presence of micro-organisms.

Previous studies with *C. briggsae* have concentrated, for the most part, on the problem of finding a chemically defined medium with which to determine its nutritional requirements. In most of the defined media tested the worms have failed to grow, but in several experiments freshly hatched worms have grown to maturity and produced a few progeny, although the growth of the progeny was very limited (Dougherty & Hansen, 1956). When certain tissue extracts were added to some of these defined media, rapid growth and reproduction followed (Dougherty & Hansen, 1956). Growth and reproduction can be sustained, probably indefinitely, by serial subculture in some of the supplemented culture media. Despite the difficulties inherent in the use of undefined tissue extracts, some insight into the nutritional requirements of the worms has been gained by the systematic omission of compounds from the supplemented basal medium. The gradual development of these techniques and certain results obtained with them have been fully discussed by Dougherty, Hansen, Nicholas, Mollett & Yarwood (1959).

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Determination of amino acid requirements presents particular difficulties because the active tissue extracts all contain much protein, which not only provides ostensibly essential amino acids, but probably also improves upon the general amino acid balance of the basal medium. The preparation and properties of the extracts have been described by Nicholas, Dougherty & Hansen (1959). So far as the need for individual amino acids is concerned, all that can be said from previous work (Dougherty *et al.* 1959) is that, when the concentration of tissue extract is reduced to such a level that only very limited growth can occur, the omission of some of the amino acids from the basal medium reduces the growth rate still further. At higher concentrations the extracts can adequately supply all the required amino acids.

To explore this problem further we have sought an alternative method of studying amino acid metabolism. We wanted to see, in particular, whether *C. briggsae* was more demanding in the number of different amino acids required than are many other animals. With this problem in mind, we have experimented with sodium acetate, labelled in the methyl group with ^{14}C, as a nutrient. We have found that the labelled carbon is incorporated into some of the worm's amino acids and believe that the experiments herein described give indirect although not definite evidence as to which of the amino acids are nutritional essentials, and which non-essentials, for *C. briggsae*.

METHODS

To determine which of the worm's amino acids become labelled with ^{14}C from sodium acetate-2-^{14}C, a hydrolysate of the worm's proteins was examined by chromatography and radioautography.

Cultures

Dougherty *et al.* (1959) have described in considerable detail our methods of maintaining axenic stock cultures of *C. briggsae*. These stock cultures provided a source of bacteriologically sterile worms with which we initiated our experimental cultures.

The culture medium used was a mixture of chick embryo extract, an autoclaved liver extract, and a solution of sodium acetate-2-^{14}C in the proportions of 2:5:3. The preparation of both the sterile chick embryo extract and the autoclaved liver extract have been described by Nicholas *et al.* (1959). The sodium acetate-2-^{14}C was dissolved in glass-distilled water, 3·3 mg./ml., and sterilized by filtration through a sintered glass filter. The three solutions were mixed aseptically, and the resulting culture medium, containing 1 mg. sodium acetate/ml., was distributed to sterile screw-capped test tubes (22 mm. diameter).

Two sets of cultures were prepared, containing sodium acetate-2-^{14}C of differing specific activity. They are referred to henceforth as the first and second experiments. In the first, a total of 11·3 ml. culture medium, containing 11·3 mg. sodium acetate-2-^{14}C with a specific activity of 8·7 μc/mg., was used. The medium was divided equally among three culture tubes. In the second, 12·9 ml. medium, containing

12·9 mg acetate with a specific activity of 28·9 μc./mg., was used, distributed to six culture tubes.

The culture tubes were inoculated with young larvae (60/ml. of culture medium) derived from egg masses. These masses, which characteristically accumulate in the stock cultures, were removed from the stock cultures, washed in four changes of buffer (M/15 potassium phosphate solution at pH 7), and then allowed to remain in the buffer overnight. A thorough account of this technique for inoculating cultures has been given by Dougherty *et al.* (1959). Because of miscalculation, there were too few larvae to complete the inoculations in the second experiment. Accordingly, the last two tubes were each inoculated with a very small egg mass taken fresh from a stock culture tube and washed in the phosphate buffer. Microscopic examination on the following day showed that the eggs had hatched, though it is likely that these tubes received rather more than 60 larvae per ml.

The cultures were incubated for 14 days at 20° C., a suitable temperature for axenic growth. Previous experience has shown that under such conditions the generation time is about 4½ days, so that three generations should have been completed in this time. Each worm, which is a self-fertilizing hermaphrodite, produces several hundred young. Examination under the microscope showed that their rate of growth was apparently normal.

Preparation of the hydrolysate

To harvest the worms, we took advantage of the fact that, although they are usually in continual motion when healthy, their ability to swim in water is poor. Only in viscous media is their swimming effective. They can therefore be washed and concentrated by sedimentation. All the cultures in an experiment were pooled and poured into a 100 ml. measuring cylinder, which was then filled with tap water and shaken. A 5 ml. sample was removed, diluted, and the worms were counted in a measured volume to provide an estimate of the numbers of worms. The worms were allowed to settle in the measuring cylinder for 4 hr. at 4° C., and then all the water except for 10 ml. was removed by aspiration, leaving the sedimented worms. By repeating the process four times we obtained worms free, as judged by microscopic examination, from any of the particulate material that collects in the cultures; accumulated egg masses were also obtained. After the final sedimentation the worms were further concentrated by low-speed centrifugation and dried to constant weight in an oven at 105° C.

The worms were then treated as described by Moses (1957) in his work on the fungus *Zygorrhynchus moelleri.* The dried mass of worms was reduced very largely to protein by extraction for 1 hr. in boiling 5% trichloracetic acid, followed by 5 min. in each of 50% aqueous ethanol, absolute ethanol, 50% ethanol in absolute ether, and three changes of absolute ether. The protein was then hydrolysed with a mixture of equal parts of 10N hydrochloric acid and glacial acetic acid, 1 ml. of the mixture per mg. of dried worms, for 18 hr. at 105° C. in a closed glass tube. The acid was removed by evaporating from water three times under an infrared lamp. Finally, the hydrolysate was taken up in 1 ml. of 3% hydrogen peroxide. Hydrogen peroxide was used to oxidize the cystine and cysteine to cysteic acid and the methionine to a mixture of the sulphoxide and the sulphone. These amino acids are more readily separated as their oxidized derivatives (Wolfe, 1957).

Two different pairs of solvent systems were employed for two-dimensional descending chromatography. These were:

(1) N-Butanol/propionic acid (71% aqueous propionic acid saturated with N-butanol) followed by water-saturated phenol (unbuffered).

(2) Butanol/ketone (N-butanol, methyl ethyl ketone, 17N ammonia, water in the proportions, 5:3:1:1) followed by butanol/propionic acid. The butanol/ketone solvent system, described by Wolfe (1957), is a monophasic system in which the solvent front is allowed to run off the paper, phenylalanine being run on a parallel paper to give a reference spot.

Oxalic acid-washed Whatman no. 4 paper was used for the separations, which were run at 20° C.

After running the chromatograms the papers were dried in a stream of air and placed in contact with sheets of No-screen X-ray film for 2–3 weeks; the films were then developed. To show up the amino acid spots, the papers were sprayed with a 0·5% solution of ninhydrin in 90% ethanol and then dried at 70° C. for 10 min. The amino acid spots were identified by comparing them with maps prepared by chromatographing 20 amino acid hydrochlorides, both individually and as a mixture, in both pairs of solvent systems. All the amino acids found in the hydrolysed worm protein were identifiable as one or other of the hydrochlorides mapped.

The radioactive amino acid spots were distinguished by the blackening of overlying areas on the X-ray film. In addition, other radioactive compounds were detected, but not identified. The radioactivity of the amino acid spots was counted on the paper with a halogen-quenched Geiger-Müller tube.

Counts were also made of measured volumes of the hydrolysate, each volume being dried on an aluminium planchette, and of worms and egg masses aseptically removed from the cultures. The worms and egg masses were thoroughly washed in five changes of water, and then dried, as groups of worms or eggs, on aluminium planchettes. Each planchette was counted with a Q-gas-filled (99·05% helium, 0·95% isobutane) G.M. tube (with a Mylar plastic end-window) and compared with counts of a standard radioactive source (i.e. a planchette coated with sodium acetate-2-^{14}C of known activity).

RESULTS

Of the two experiments, the second was a repeat of the first, except that acetate of higher specific activity was used. The second gave more information than the first, because more amino acids were detectably labelled, presumably by reason of the more radioactive acetate used therein and of a progressive improvement in chromatographic techniques. Thus, the earlier work had given the basis for approximating, in the second experiment, to an optimal volume of protein hydrolysate to be applied (a volume equivalent to about 1·5 mg. dry material—worms and egg masses—gave best results).

A surprising difference, however, was found in the combined dry weight of worms and egg masses recovered from the two experiments, i.e. 6·8 mg. in the first compared with 2·7 mg. in the second. This discrepancy was too large to be accounted for by possible deviation in volume or constitution between the two culture media. Nor could it be accounted for in differences in the inoculum, although, as we noted in describing our methods, it was found necessary to inoculate two of the second experiment's six tubes with fresh egg masses instead of young larvae. Examination of the cultures under the microscope showed that the reproduction was less in all tubes of the second experiment—and least of all in the two tubes inoculated with egg masses. The growth rate, for at least the first generation, was normal, maturity being reached in about 4½ days. In the absence of appropriate experiments it is impossible to say whether the differing levels of radioactivity in the first and second experiment could have affected the yield of worms or eggs.

It was estimated from counts of diluted samples that the 6·8 mg. recovered in the first experiment represented about 60,000 worms at all stages of growth, as well as many large egg masses, each containing many thousands of eggs. It is important to emphasize that the protein hydrolysate included both worm protein and protein derived from substantial quantities of accumulated eggs. In both experiments only a very small proportion of the ^{14}C added to the culture medium was recovered in the protein hydrolysates, 0·088% in the first experiment and 0·026% in the second. It was none the less found possible to count the activity of individual worms and egg masses (see Table 1).

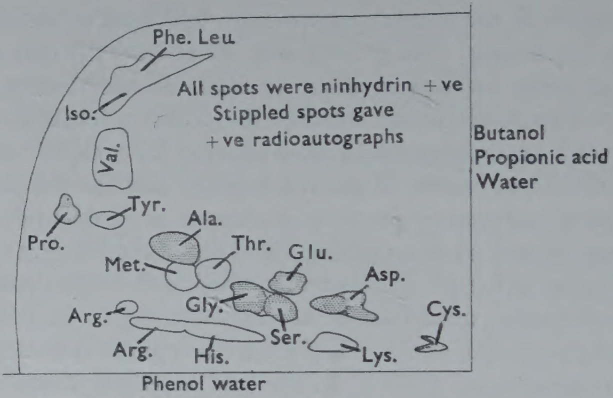
Table 1. *The radioactivity of worms and their egg masses removed from sodium acetate-2-^{14}C cultures (2nd experiment)*

	Age of culture (days)	No. counted	Mean disintegrations/minute*	Mean activity
Adult worms	6	5 worms	85/worm	$3{\cdot}8 \times 10^{-5}$ μc./worm
Adult worms	12	10 worms	184/worm	$8{\cdot}2 \times 10^{-5}$ μc./worm
Egg masses	6	Several hundred eggs	Not significantly above background	Not measurable
Egg masses	12	Several hundred eggs	4,800/mass	$2{\cdot}2 \times 10^{-3}$ μc.

* Corrected for efficiency of G.M. tube; mean background activity subtracted.

In the first experiment the following amino acids were found on the paper chromatograms (with use of both pairs of solvent systems): alanine, arginine, aspartic acid, cysteic acid, glutamic acid, glycine, histidine, lysine, proline, serine, threonine, tyrosine, valine, and the combined spot for leucine, isoleucine and phenylalanine. All these amino acids and, in addition, methionine, were found in the second experiment. Methionine was only present in small amounts, and no doubt was found in the second experiment because of better technique. Tryptophane, the remaining amino acid commonly found in proteins, would be destroyed by the method of hydrolysis used.

In the first experiment, radioautographs of the chromatograms showed alanine, aspartic acid, glutamic acid, and glycine to have become labelled. In the second experiment, alanine, aspartic acid, cysteic acid, glutamic acid, glycine, proline, and serine were labelled with ^{14}C. Finally, we were able to incorporate all these findings in a single chromatogram together with its accompanying radioautograph. They are illustrated in Text-fig. 1, and Pl. 8. The relative activities of the amino acid spots in this chromatogram are given in Table 2.



Text-fig. 1. Tracing of chromatogram of *C. briggsae* amino acids (Exp. 2).

Table 2. *The proportional radioactivity of the amino acids labelled with ^{14}C in the protein hydrolysate (2nd experiment)*

Amino acid	Counts/min.* (mean of both sides of paper)	% total amino acid ^{14}C†
Aspartic acid	240	42
Glutamic acid	181	32
Glycine	18	3·2
Serine	14	2·5
Proline	41	7·2
Alanine	61	11
Cysteic acid	12	2·1
Methionine	0	0
Origin	25	—
Background (mean of 5 counts)	43	—

* With mean background count subtracted, but not corrected for paper absorption or efficiency of the G.M. tube—factors that would remain constant throughout (self-absorption is considered negligible).

† About 14% of the ^{14}C activity of the hydrolysate is represented by the activity of the amino acid fraction.

DISCUSSION

The hypothesis that we wish to discuss further is that the labelled amino acids can be synthesized by the worms and that they are not, therefore, required in the diet, provided that the diet is adequate in other respects. It must be emphasized that although the worms no doubt ingested some of the ^{14}C as sodium acetate-2-^{14}C, this compound was not, in all probability, the only form in which the ^{14}C was taken in, because the ^{14}C label may well have been excreted in a variety of compounds, only to be taken up again by the worms.

So far as we are aware, the only evidence against this hypothesis comes from work on the incorporation of labelled metabolites into mouse brain by Rafelson, Winzler & Pearson (1951), Winzler, Moldave, Rafelson & Pearson (1952), Moldave, Winzler & Pearson (1953) and Moldave, Rafelson, Lagerborg, Pearson & Winzler (1954). These workers were primarily interested in the effects of virus multiplication on the metabolism of the mouse brain and worked, for the most part, on preparations of minced mouse brain taken from the day-old mouse. They found to their surprise that ^{14}C from uniformly labelled glucose was incorporated into all the amino acids, both essential and non-essential for the mouse, except threonine and proline (Rafelson *et al.* 1951). Following up this discovery they showed that ^{14}C from uniformly labelled glucose was incorporated into the same amino acids in the intact mouse (Winzler *et al.* 1952) but that the brain of the day-old mouse was apparently unusual in this respect for in the day-old mouse liver and intestine and the adult mouse brain, both *in vivo* and *in vitro*, ^{14}C was only incorporated in some of the non-essential amino acids (Winzler *et al.* 1952). Minced mouse brain from the day-old mouse also incorporated ^{14}C from other labelled metabolites into the amino acids (Moldave *et al.* 1953) including several of the essential amino acids. The evidence suggests that the ^{14}C is incorporated first into free amino acids and that labelled protein-bound amino acids are derived from the labelled free amino acids (Moldave *et al.* 1954). Most of the ^{14}C derived from glucose was incorporated in the carboxyl groups of the amino acids (Rafelson *et al.* 1951, and Moldave *et al.* 1953).

Support for the hypothesis comes from the work of Black, Kleiber, Smith & Stewart (1957), who found that the lactating cow incorporated ^{14}C from labelled acetate into the non-essential amino acids secreted as casein, but not into lysine, chosen as a representative of the essential amino acids (for the cow). If, as we think reasonable, the labelling of one of the amino acids is taken as evidence that it can be synthesized by *C. briggsae*, it remains to be demonstrated that it can be synthesized at an adequate rate to sustain growth and reproduction. A final solution to this problem must probably await the development of a defined medium for culture experiments. However, experiments by Dougherty *et al.* (1959) suggest that in a minimal culture medium, i.e. one which will just support continued reproduction, containing the essential amino acids, as defined below, either glycine, or glutamic acid, or both, are capable of replacing all the other non-essential amino acids, although other experiments suggested that none of the amino acid mixtures was optimal. The culture medium also contained suboptimal levels of liver protein.

It cannot be assumed that none of the unlabelled amino acids can be synthesized. In fact, changes in cultural conditions might increase the number becoming labelled, just as, for example, raising the specific activity of the acetate in the second experiment compared with that used in the first experiment increased the number of labelled amino acids that were detected. The proportion of the ^{14}C recovered might have been considerably increased by changing the culture medium in other ways;

for example, Roberts, Abelson, Cowie, Bolton & Britten (1955), found the uptake of labelled acetate was greatly influenced by the presence of glucose in cultures of *Escherichia coli*.

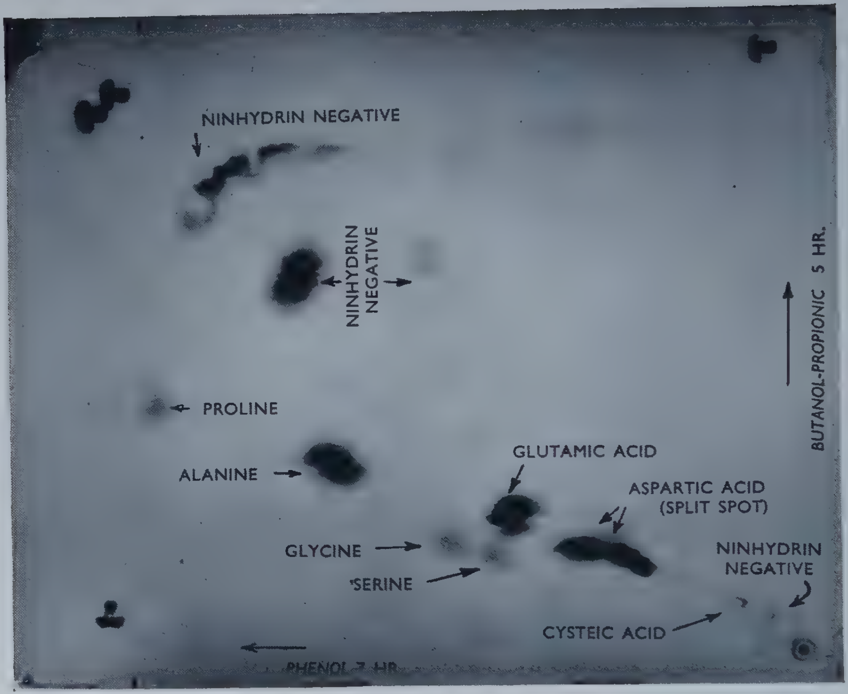
Here too, it will be profitable to consider the results of culture experiments by Dougherty *et al.* (1959). As was noted in the Introduction to this paper, the omission of certain individual amino acids from a defined basal medium reduced the rate of growth. It will be recalled that the effect of the omissions was only noticeable in media in which the protein-rich supplement had been reduced to a level less than that required for satisfactory growth. It is interesting to note that the amino acids giving this result were the nine, excluding tyrosine, that did not become labelled in either of our experiments—plus tryptophane (destroyed in hydrolysing the worm protein). The culture experiments, therefore, suggest that these ten are required, but what they cannot tell is whether, at higher growth rates, additional amino acids might not be required.

Despite the limitations of our method, it is interesting to compare the nutrition of *C. briggsae* with that of other animals, so far as it is known, on the assumption that the unlabelled amino acids are essential in the diet and the labelled amino acids are not. Two qualifications should be made, both on grounds of comparative nutrition and in the light of the experiments by Dougherty *et al.* referred to above. Tryptophane, destroyed by acid hydrolysis and therefore not present in our chromatograms, should be added to the list of essential amino acids, and tyrosine, known to be synthesized by other animals from phenylalanine, omitted. The list of essential amino acids thereby derived i.e. arginine, histidine, leucine, lysine, isoleucine, methionine, phenylalanine, tryptophane, threonine and valine would include those known to be required by most of the few animals whose amino acid requirements have been worked out in any detail. Reference should be made to Meister (1957) and Kidder (1953) for a detailed discussion of the amino acid requirements of other animals. *C. briggsae* is apparently similar to some strains of the ciliate *Tetrahymena pyriformis*, the beetles, *Tribolium confusum* and *Attagenus* sp. and the rat. It is thus more demanding than the mouse and dog, which do not need arginine, but less demanding than other strains of *Tetrahymena pyriformis*, the flagellate *Trichomonas foetus*, the mosquito *Aedes aegypti* and the domestic fowl, all of which require additional amino acids.

SUMMARY

1. The nematode, *Caenorhabditis briggsae*, was cultured axenically in a mixture of chick embryo extract, autoclaved liver extract and sodium acetate-2-^{14}C. A protein hydrolysate was prepared from the worms and the eggs which were collected from the cultures.

2. Chromatography and radioautography were carried out in a study of the amino acid composition of the hydrolysate. The following amino acids were found labelled: aspartic acid, glutamic acid, alanine, proline, glycine, serine. Cystein and cystine were oxidized to cysteic acid which was also labelled. The following amino acids



NICHOLAS, DOUGHERTY, HANSEN, HOLM-HANSEN AND MOSES—THE INCORPORATION OF ^{14}C FROM SODIUM ACETATE-2-^{14}C INTO THE AMINO ACIDS OF THE SOIL-INHABITING NEMATODE, *CAENORHABDITIS BRIGGSAE*

(Facing p. 443)

were not labelled: arginine, histidine, lysine, methionine, threonine, tyrosine, valine and the combined spot representing phenylalanine, leucine and isoleucine. Tryptophane would have been destroyed by our method of hydrolysis.

3. Since the labelled amino acids are synthesized by the worm, it is suggested, tentatively, that they are not required in an otherwise adequate diet. So far as the unlabelled amino acids are concerned, it is suggested, on the basis of the results of certain culture experiments (published separately) that, with the probable exception of tyrosine, they are essential in the diet of *C. briggsae*.

We wish to thank Prof. Melvin Calvin of the University of California for supplying us with labelled sodium acetate-2-^{14}C and for the use of facilities in his laboratory.

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EXPLANATION OF PLATE

Radioautograph of a protein hydrolysate of *Caenorhabditis briggsae* which had been allowed to feed on sodium acetate-2-^{14}C.

THE DETERMINATION OF TOTAL CARBON DIOXIDE IN WATER

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INTRODUCTION

Compared with oxygen the estimation of carbon dioxide in water presents much greater difficulties, and the methods published until now do not approach in sensitivity the Winkler method for dissolved oxygen by which the latter can be measured to 0·02 mg./l. in unpolluted and 0·2 mg./l. in polluted natural waters (Wheatland & Smith, 1955, Beadle, 1958). This is partly due to the high solubility and rate of diffusion of carbon dioxide in water which makes it difficult to avoid a significant change in concentration when sampling or during the process of estimation. Those methods which depend upon measurement of pH are particularly subject to errors arising from this factor, and the sensitivity of any measurement of pH is in any case a factor limiting accuracy. In polluted natural waters, as in swamps or water in which the respiration of organisms in low oxygen concentrations is being studied, the presence of acids other than carbonic may also introduce errors in methods relying on measurement of pH.

The method described in this paper was designed to measure the concentration of total carbon dioxide in the swamp waters of Uganda and to be used in conjunction with a constant flow respirometer where oxygen consumption and carbon dioxide production by aquatic organisms were measured simultaneously. In order to obtain meaningful results with the respirometer, it was found necessary to determine the total carbon dioxide concentrations of the effluent water samples to a degree of accuracy not readily achieved by any of the published methods (see Table 1 for comparative data). At the same time as these experiments were in progress, attention was directed to the carbon dioxide content of the waters which formed the natural habitat of the experimental material. This method has proved to be suitable, not only for determinations on these waters where the total concentration may be as high as 180 mg./l., but also for rain water where the range may be only 1–2 mg./l.

PRINCIPLE OF THE METHOD

All gases in solution are removed from an acidified sample by the vacuum extraction technique of van Slyke & Neill (1924) and are then transferred without contact with the atmosphere to a standard volume of sodium hydroxide solution with a known electrical resistance at a standard temperature. Within a given range of carbon dioxide content, the change in resistance of the absorbent after equilibration with the gas bears a strictly linear relationship to the total carbon dioxide content of the sample. The apparatus has been calibrated by using a series of standard test solutions of sodium carbonate.

The time required for a single determination is 15–20 min.

RANGE AND ACCURACY

The concentration of total carbon dioxide is expressed throughout in terms of mg. per litre but the actual quantity measured is obviously determined by the volume of the sample. The volume of 10 ml. which was eventually adopted is a compromise value of which (*a*) the upper limit is governed by a 1 : 10 relation between the sample volume to the volume of the extraction chamber to ensure that most of the dissolved gas will be extracted (van Slyke & Neill, 1924), and (*b*) the lower limit, below which there are significant errors in measuring the sample.

Thus the method has been used for quantities of total carbon dioxide within the ranges of 0·02–0·5 mg. *and* 0·5–2·0 mg./10 ml. sample, though it is necessary to use absorbent solutions of different strengths to obtain the maximum accuracy for each range. This is achieved in theory by adjusting the strength of the absorbent to equate with the highest value of carbonate concentration likely to be encountered. In practice, however, the strength of the absorbent is best adjusted to a fairly wide range of carbon dioxide values and we found the 0·005 N solution very suitable for concentrations up to 50 mg./l.

A maximum variation of 2 Ω was noted for the final resistance values of the absorbent from five replicate determinations on the same solution during a routine calibration of the apparatus for total carbon dioxide values up to 50 mg./l. Many subsequent determinations showed this variation to be a maximum, though it was often less, and a series of critical determinations, performed after more experience with the technique had been gained, showed that even more exact replication was possible and the performance of the bridge then became a limiting factor in the accuracy of the method. The final resistance of the absorbent was constant to 1 Ω when replicate determinations were performed on a standard carbonate solution containing 36·0 mg./l. carbon dioxide and on solutions containing amounts as low as 5·2 mg./l. Ordinarily the coefficient of variation is 0·2 % for any concentration within the 0–50 mg./l. range, but the percentage error is inversely proportional to the concentration. Greater accuracy could be achieved at the lower concentrations by reducing the strength of the absorbent which would in turn limit the range.

APPARATUS

The apparatus consists of the following main parts:

(*a*) Vacuum extraction unit (Fig. 1).
(*b*) Gas absorption unit for use with (*a*) (Fig. 2).
(*c*) Conductivity cell (Fig. 3).
(*d*) Sensitive a.c. bridge for resistance determinations of the absorbent.

Table 1. *Comparative data on methods for the determination of carbon dioxide in water*

Type of method	No.	Author and reference	Free or total CO_2	Sample size	Accuracy	Suitable range of concentration	Time required for one determination	Main sources of error
	1	Conway (1950)	Total	0·2 ml. 1·6 ml.	0·5 % coefficient of variation	No. 1 unit down to 0·2 mg. No. 2 unit down to 0·04 mg.	50–60 min.	CO_2 of the air affects accuracy of titre when concentration of CO_2 is low in the sample
Evolution of gas followed by absorption in standard alkali	2	Partridge & Schroeder (1932)	Total	100 ml.	p.p.m. Known Max. error 8·8 −0·2 44·0 −2·5 87·0 −2·0	Tested up to 87 p.p.m.	20 min.	Presence of volatile acids other than CO_2; titration errors
	3	Larsen (1949)	Total	(Normally solid)—up to 76 mg.	mg. Known Max. error 1·211 +0·006	0·2–2·0 mg.	30 min.	Titration errors; variation in CO_2 content of air; salts of volatile acids in sample
Titration methods	4	McKinney & Amorosi (1944)	Total	50 ml.	p.p.m. Known Max. error 51·0 −0·3 6·0 +0·6 516·5 +2·3	Tested up to 516·5 p.p.m.	20–25 min. less for a series	Exchange of free CO_2 with atmosphere; salt effect on indicators; presence of acids other than CO_2
	5	Moore (1939)	Free	25 ml.	Depends on accuracy of pH measurement—0·05 pH unit ≡ 10–15 mg. CO_2 at 0·0015 N alkalinity	—	10 min.	Inaccurate pH measurement; loss of free CO_2 to atmosphere; presence of acids other than CO_2
Modified Winkler	6	Review by Partridge & Schroeder (1932)	Total	Variable	Low in presence of sulphate or hydroxide	Tested up to 86 mg./l.	—	Exchange of free CO_2 with atmosphere; presence of sulphate or hydroxide; indicator adsorbed onto carbonate particles

Table 1 (*cont.*)

Phenolphthalein-methyl orange method	7	Review by Partridge & Schroeder (1932)	Total	Variable	Low in presence of hydroxide and other interfering subs. p.p.m. Known — Max. error 6·0 — +18·7 46·1 — +10·9	Tested up to 86 mg./l.	—	Exchange of free CO_2 with atmosphere; interfering substances e.g. weak acids; hydroxide, sulphate
Vacuum extraction followed by:								
(*a*) Manometric measurement	8	van Slyke & Neill (1924)	Total	0·5 ml. 1·0 ml. 2·0 ml.	mg./l. Known — Max. error 220 — +2·0 440 — +2·0 660 — +2·0 1320 — +2·0 1980 — +6·0	Tested from 220–1980 mg./l.	15–20 min.	Reading errors of the manometer; variable rate at which gas is reabsorbed by the sample
(*b*) Manometric measurement (modified van Slyke)	9	Oesting (1934)	Total	10 ml.	mg./l. Known — Max. error 45·36 — +0·5 45·98 — +1·0 47·04 — −0·1	Tested from 45–59 mg./l.	15–20 min.	Similar to (8)
(*c*) Volumetric measurement (modified van Slyke)	10	McLendon (1917)	Total	10 ml.	mg./l. Known — Max. error 137·4 — −1·5	Tested *c.* 138 mg./l.	—	Variable rate of reabsorption in relation to salinity of sample; corrections necessary for temperature and barometric pressure
(*d*) Conductivity measurement	11	Our method	Total	10 ml.	0·2 % coeff. var. or ±0·15 mg./l. up to 50 mg./l. total concentration	Tested from 4 to 50 and 50 to 200 mg./l.	15–20 min.	Handling of samples; error of sample measurement; variable rate of reabsorption; incomplete transfer of gas

(*a*) *The gas extraction unit and sample pipette* (Fig. 1)

This is basically similar to the apparatus of van Slyke & Neill, but there are some modifications. The size of the sample (10 ml.) is larger than that normally used, and therefore the dimensions of the extraction chamber have been increased accordingly to about 130 ml. The latter consists of a stout round-bottomed flask connected to the manometer and taps T_4 and T_5 by means of a ball-and-socket joint. The sample in the extractor chamber is stirred by a magnet (*F*) sealed into stout Pyrex tubing and actuated by a second rotating horseshoe magnet (*M*) placed near it.

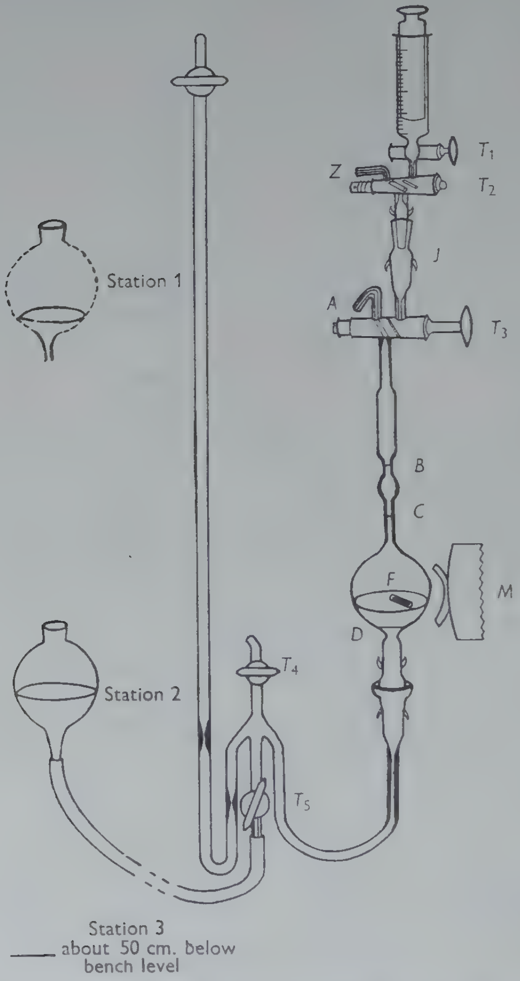

Fig. 1. General layout of gas extraction apparatus with magnetic stirrer. The rotating magnet *M* shown to the side of the extraction chamber is more conveniently placed behind it. The absorption unit is shown in position. *A* is about 10 cm. long and 2 mm. bore. T_2, T_3, T_4 and T_5 are van Slyke stop cocks. For explanation see text.

The sample is introduced into the extraction chamber through the inlet tube *A* and is contained in that part of the extraction chamber above the upper graduation mark *B*. The lower graduation mark *C* permitted a standard quantity of orthophosphoric acid to be drawn into the pipette *A* and 2 ml. were adequate to displace the sample from *A*. Tap T_3 should be of the standard high-quality van Slyke type with a properly aligned plug, so as to allow complete transference of the gas (see 'Manipulation').

(*b*) *Gas absorption unit* (Fig. 2)

This consists essentially of a 20 ml. all glass syringe connected to a 3-way tap (T_2), of the same type and quality as T_3, with a piece of 1 mm. bore capillary tubing. The unit is connected to the extraction unit by a B 10 standard cone, the tip of which should be ground on the inside to reduce the thickness of the glass and thus to ensure that the gas is not trapped between the mercury and glass when the transfer is made (Fig. 2). The single way tap (T_1) provides an additional outlet which is used both when filling the unit with sodium hydroxide solution and when transferring it to the conductivity cell after equilibration with the gas.

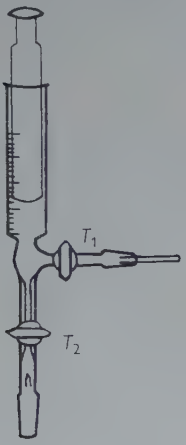

Fig. 2

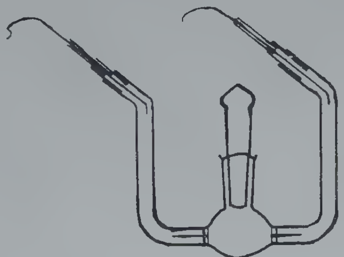

Fig. 3

Fig. 2. Absorption unit, seen from direction at right angles as compared with Fig. 1.
Fig. 3. Conductivity cell.

(*c*) *Conductivity cell* (Fig. 3)

This is of standard design and the dimensions are not critical (Jones & Bollinger, 1931). For maximum sensitivity, however, the plates must not be too large or too close together. Our home-made cell has circular platinum foil plates cut out with a no. 2 cork borer and placed 22 mm. apart. These plates, which are finally blackened (Jones & Bollinger, 1935), are sealed rigidly on to the ends of the side arms. Platinum wires connect the plates through mercury and platinum connexions to the bridge.

The cell was closed, excluding air bubbles, with a B10 stopper to prevent contamination of the absorbent by atmospheric carbon dioxide. The constancy of the cell was checked with a standard sodium chloride solution.

(*d*) *A.C. bridge for resistance determinations*

All resistance determinations were made at 30° C. when the cell and contents had equilibrated to this temperature in a water-bath. The temperature was constant to 0·01° C.

The a.c. bridge was of standard design, comprising non-inductively wound ratio arms and variable resistance to 10,000 Ω, Mullard oscillator (1000 c.p.s.) and headphones, and incorporated variable condensers (0·005 mF) symmetrically placed in the circuit opposite the cell. These latter were adjusted to compensate for the capacitance of the cell, the value of which varies with the resistance of the solution. By this means it is possible to obtain a sharp end point.

ABSORBENT

For the range of total carbon dioxide concentration usually required (0–50 mg./l.), the most convenient absorbent was found to be 0·005 N (approx.) sodium hydroxide solution. This was prepared by diluting a clear saturated solution of the alkali with relatively carbon-dioxide-free distilled water.* An approximately normal solution was first prepared by diluting 55 ml. to 1 l. and 50 ml. of this stock solution were then diluted to 10 l. with carbon-dioxide-free distilled water prepared by aeration for several hours. The final strength of the solution is about 0·005 N, with a carbonate content of 1 mg./l., and the resistance measured by our cell was approximately 2730 Ω. With a little practice it is quite possible to prepare successive stocks of absorbent which will have practically the same resistance value. The minor variations between different batches of the absorbent, which affect the accuracy of the results, can be adjusted by the method given below without the frequent necessity of re-calibrating the apparatus.

The absorbent was protected from contamination by atmospheric carbon dioxide by a caustic soda trap and mercury-to-glass seals. In spite of these precautions, however, the resistance of the solution increased by about 3 Ω per day.† Since this drift will cause significant errors if corrections are not applied, the resistance of the absorbent was measured before each series of determinations and the increase over the value of the original calibration was noted. This increase was then subtracted from the final values obtained for each determination and fixed base-line was thus retained. The correctness of this method was tested by varying the resistance of the absorbent by adding water or sodium hydroxide solution and subsequently performing a series of determinations on two standard carbonate solutions with a total carbon dioxide content of 29·0 and 22·8 mg./l. Table 2 shows that the determinations agree within the limits of the method, irrespective of the variations in resistance of the absorbent solution.

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* Prepared by aerating conductivity water for several hours. Complete removal of the carbon dioxide by this means is not possible and water treated in this way contained 0·8 mg./l. (Calculated value is 0·3 mg./l. at 24° C. and 660 mm. barometric pressure.)

† The carbonate concentration was constant over a period of 14 days, but the resistance increased by 41 Ω, presumably due to a reaction between the alkali and glass container.

The strength of the absorbent solution was increased when the samples contained more than 50 mg./l. and a 0·02 N solution was found to be satisfactory for values up to 200 mg./l. The resistance of this solution was 730 Ω in our cell and the resistance determination then becomes a factor limiting the accuracy of the method. By using a fractional Ω box, replicate determinations on solutions containing 180 mg./l. total carbon dioxide differed by no more than 1 mg./l. The errors incurred in handling samples such as this which contain a very high concentration of free carbon dioxide may, in fact, mask the errors of measurement.

Table 2. *Carbon dioxide content of two standard carbonate solutions determined by using absorbent solutions of differing strengths*

(Strength expressed as resistance measured at 30 °C.)

CO_2 content (mg./l.) test solution (prepared)	Initial R_{30} absorbent (A)	Final R_{30} absorbent (B)	Difference (Ω) (B − A)	CO_2 content (mg./l.) (determined)
A 29·0	2724	2906	182	29·0
29·0	2767	2951	184	29·2
29·0	2762	2954	182	29·0
B 22·8	2797	2936	139	22·0
22·8	2790	2927	137	21·7
22·8	2782	2921	139	22·0
22·8	2772	2911	139	22·0
22·8	2764	2902	138	21·8

When there is no previous indication of the range of carbon dioxide concentration in a series of samples, it is advisable to make a preliminary measurement using a relatively strong solution of sodium hydroxide (e.g. 0·02 N). If there is less than 50 mg./l. carbon dioxide and greater accuracy is required, the weaker (0·005 N) solution can then be used.

During preliminary experiments barium hydroxide solution was used as the absorbent, but it was rejected on finding that the carbonate precipitate caused erroneous resistance values, presumably by settling on the plates of the cell. Thomas (1933) does not appear to have experienced this trouble, but we found that stable readings could be obtained only when the precipitate was filtered off under carbon-dioxide-free conditions. This was abandoned for the greater convenience of sodium hydroxide.

MANIPULATION

Cleaning procedure

(*a*) *Extraction unit.* Traces of sample remaining from the previous determination can be removed by the following procedure:

(1) Close control cock (T_5) and open T_3 to *J*.

(2) Raise mercury reservoir to station 1.

(3) Open T_5 slowly until the mercury just enters *J*. Close T_5.

(4) Remove liquid from the walls of *J* and the surface of the mercury by means of a small plug of cotton-wool twisted round a spill.

(*b*) *Absorption unit.* Mercury is removed from the bore of T_2 and the whole unit is washed with conductivity water, rinsed with acetone and dried with a jet of compressed air.

Assembly

The absorption unit is filled with the solution by closing T_2 and flooding the syringe by means of a plastic tube connecting T_1 to the aspirator. The plunger is then inserted for a short distance while the liquid is flowing and T_1 is then closed. The volume of the absorbent is then adjusted to a standard but undetermined value (20 ml. approx.) by means of a 'stop' on the plunger with T_1 in the open position. The absorbent will not flow into the capillary connexion between T_2 and the syringe. This avoids the risks of contamination when the transfer of gas is made from the extraction chamber to the unit.

With the foregoing technique it is possible to transfer the absorbent from the aspirator to the unit without contamination by atmospheric carbon dioxide.

The cone at the base of the unit is greased slightly and inserted firmly into *J*. Springs are attached. With T_2 open to *Z* the whole of the space below T_2 is filled with mercury by manipulation of T_2, T_3, and T_5, with the mercury reservoir at station 1. The sample inlet tube (*A*) is filled to the tip by reversal of T_3, when the mercury flow is controlled by T_5.

The sample is now introduced, acidified, the gases removed by vacuum extraction and these are transferred to the absorption unit. The details of the manipulation are given below.

Introduction of sample and acid

(1) With control cocks T_5 and T_2 closed and T_3 open to *A*—place the mercury reservoir at station 2.

(2) Insert *A* into sample bottle (30 ml. capacity) so that the tip reaches about half way down.

(3) *Partially* open control cock T_5 *gently* and draw in the sample until the mercury meniscus reaches the graduation mark *B* (10 ml.).

(4) Close T_5—remove bottle and wipe off excess sample from *A*.

(5) Place *A* into a small bottle of undiluted orthophosphoric acid* and open T_5. Draw in 2 ml. to acidify and displace the sample from the capillary and bore of the cock. Close T_3 quickly to avoid mixing of the sample with the acid in *A*. The pH of the mixture is about 1·2.

Operations 2 and 3 must be performed with *no delay* once the sample has been opened, especially when it contains free carbon dioxide.

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* The error due to interfering substances in the orthophosphoric acid is very small, being equivalent to about +0·05 mg./l. carbon dioxide, but the same amount of undiluted lactic (used as a 1 N solution in the usual van Slyke procedure) can cause errors up to +3·5 mg./l., presumably due to the presence of a volatile acid.

Extraction procedure

(1) Lower the mercury reservoir to station 3 and, when the liquid meniscus level has reached the region of B, open T_3 to J and clear it of mercury.

(2) Close T_5 when the mercury and sample reach a fixed point (D) at the base of the extraction chamber (Fig. 1). Extract for 5 min. with continuous stirring.

Transfer of gas

(1) Raise the mercury reservoir to station 1, and *partially open* the control cock (T_5) so that the mercury and sample rise, *rapidly* but without turbulence.

Control the rate at which the sample rises by manipulation of T_5 and *decrease the rate* as soon as the liquid meniscus enters the narrow tube below T_3. *Close* T_3 *quickly* when liquid is seen just about to enter the bore of the cock.

The gas is now confined in J under negative pressure.

(2) Open T_3 to A and clear the pipette of liquid.

(3) Partially reverse T_3 and allow mercury to enter J steadily. A small volume of liquid may also enter but will be dispersed rapidly over the relatively dry walls of J. The surface of the mercury will eventually appear to be dry.

(4) *Partially open* T_2 to the syringe and transfer the gas. The mercury will finally fill the bore of the cock, thus clearing the gas, but will not pass into the capillary tube above. This operation is made easier if the upper extremity of the bore faces the operator. Close T_2.

(5) Lower the reservoir to station 2 and open T_2 to the outside.

Resistance determination

(1) Detach the absorption unit and shake thoroughly to ensure complete absorption of the gas and to homogenize the contents.

(2) Clamp the unit on its side with T_1 facing downwards and flood out the conductivity cell by forcing the absorbent out steadily through T_1 and a plastic tube connexion, by means of the plunger.

(3) Stopper the cell quickly (dry stopper) and determine the resistance of the solution after equilibration in a water-bath at 30° C., indicated by two constant readings—about 5 min.

CALIBRATION

A series of twelve standard sodium carbonate solutions with carbon dioxide contents ranging from 9·6 to 124·0 mg./l., was prepared from the A.R. solid (preheated at 270° C. overnight) and relatively carbon-dioxide-free distilled water prepared by aeration for several hours.*

Duplicate determinations were then performed on each sample with 0·005 N-NaOH as absorbent and using the procedure outlined in 'Manipulation'. Fig. 4 shows the strictly linear relationship between the total carbon dioxide content of the sample and the resistance of the sodium hydroxide solution for values up to 52 mg./l. carbon dioxide. The slope changes when the total carbon dioxide content of the sample exceeds this value. A possible explanation is that the two slopes reflect the two equilibrium systems, (1) —OH^-/–$CO_3^=$ and (2) —$CO_3^=$/–HCO_3^-.

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* The total carbon dioxide content of this distilled water was 0·8 mg./l. and this amount is included in the total value for each standard solution.

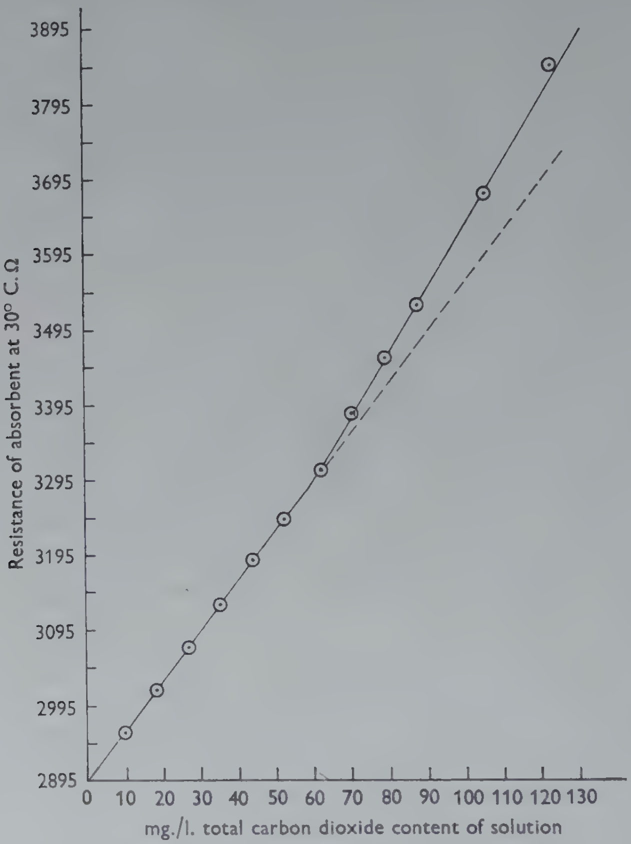

Fig. 4. The relation between total carbon dioxide content of standard carbonate solutions and the final resistance of the absorbent (0·005 N sodium hydroxide solution) at 30° C.

The carbon dioxide concentrations produced in our respirometer did not exceed 50 mg./l. and thus the lower portion of the curve could be used.

Large-scale graphs can be constructed from measurements on standard sodium carbonate solutions to cover any desired range using the appropriate sodium hydroxide solution. In practice, however, we have found that 0·005 N-sodium hydroxide for carbon dioxide concentrations up to 50 mg./l. and 0·02 N for those up to 200 mg./l. were suitable for our particular purposes in respirometry and the study of natural swamp waters. The errors introduced through subsequent small variations of the resistance of the absorbent can be corrected without re-calibration, providing that the conductivity cell constant does not change (see 'Absorbent').

The initial calibration described above has established the linear relationship between resistance of the absorbent and the carbon dioxide content of the sample. Three standard carbonate solutions are therefore in fact sufficient to construct the graph.

SOURCES OF ERROR

There are three main sources of error: (A) those incurred by incorrect handling of the samples and absorbent, (B) manipulative errors of the actual determination, and (C) interfering substances.

(A) *Errors in handling of samples and absorbent*

(1) *Samples*

Significant errors can be introduced if samples and the absorbent are handled in such a way as to permit exchange of carbon dioxide with the atmosphere. This is particularly liable to occur where weakly buffered samples contain a relatively high concentration of the 'free' gas. Such solutions should be stored under water in glass bottles with well-fitted glass stoppers. The transfer of samples from one type of receiver to another should always be done by siphoning slowly with the tube placed near the bottom of each. The receiving vessel should be flooded out with a large excess of liquid.

(2) *Absorbent*

(i) The weak solution of sodium hydroxide is easily contaminated by atmospheric carbon dioxide, and therefore the absorption unit must be filled by the flooding technique outlined in 'Manipulation'. The well-fitting plunger of these syringes excludes atmospheric carbon dioxide, at least during the period required to complete a determination.

(ii) Similar considerations apply to the transference of the absorbent from the unit to the conductivity cell. The 20 ml. capacity of the unit is adequate to flood out the cell previously filled with distilled water. Errors were introduced when the volume of the absorption unit was reduced to 10 ml.

(iii) The stock absorbent should be agitated before use and it is advisable to run 10–20 ml. to waste before a determination is started, particularly if it has been standing unused for several days.

(iv) The carbon dioxide is absorbed rapidly by the solution in the unit but the contents must be equilibrated. 'Dead spots' near the taps may be cleared by manipulation of the gas bubbles.

(B) *Errors in manipulation*

(1) *Sample volume*

Significant variation may be caused by the presence of residual sample from previous determinations lining the walls of the pipette and related parts, as a thin film. If the glassware is relatively free from grease and the sample is cleared slowly, then liquid is rarely trapped between the mercury and the glass.

(2) *Reabsorption of gas after extraction*

The manometric method of van Slyke & Neill (1924) (van Slyke & Sendroy, 1927) uses tables of factors which compensate for the reabsorption of carbon dioxide by the samples during the period after extraction when the mercury is adjusted to the datum line. Accurate results therefore depend on careful timing and adjustment of the mercury level. We have found that a significant error due to this factor is eliminated, if the operations of transferring the gas to *J* and later to the absorbent are done within about $1\frac{1}{2}$ min. An error of about $-0{\cdot}6$ mg./l. was introduced when the time was extended to 4 min.

It might be expected that the film of liquid left on the walls of *J* during the extraction procedure (stage 1) would reabsorb some carbon dioxide when the pressure is increased, but (*a*) the pH of this residual liquid is very low (1·2), being derived from a previous acidified sample, and (*b*) the volume is extremely small, and (*c*) the gas remains in contact only for a few seconds. The results from standard solutions show that there is in fact no significant reabsorption at this stage.

(3) *The introduction of mercury into the absorption chamber*

This will rarely occur if T_2 is only partially opened during the transfer of gas. The mercury will be contaminated with the contents of the extraction chamber and will thus cause faulty resistance values.

(C) *Interfering substances*

Non-volatile substances such as phosphate and hydroxide which interfere with titration methods do not affect the accuracy of this method but volatile acidic substances such as hydrogen sulphide will be transferred with the carbon dioxide and be taken up by the absorbent. The 'apparent' total carbon dioxide would then be in excess of the real value.

Our swamp water samples have so far contained no significant amount of sulphide, but if such were ever the case it could perhaps be removed by the insertion of a crystal of soluble mercury or cadmium salt into the original sample on collection. The sulphide precipitate must then settle before a sample is taken off into the apparatus. Methane, which is always present in swamp waters, does not of course interfere with the method.

DISCUSSION

Table 1 reviews the various methods used for the determination of total carbon dioxide and one method (Moore, 1939) for 'free' (dissolved) carbon dioxide. They vary greatly with respect to the volume of sample required, the range of concentration for which they were designed, and the accuracy. Since our method has been designed primarily for use with natural waters where the amount of sample is rarely limited, the elegant method of Conway (1950) will not be considered as it was designed for use with small volumes of sample containing relatively high concentrations of carbon dioxide.

Titration methods (Table 1; 4, 5, 6 and 7) offer certain advantages in that little specialized equipment is required, but they rarely achieve a desirable degree of accuracy due to the exchange of carbon dioxide with the atmosphere, particularly when the sample is weakly buffered and interfering substances such as hydroxide, phosphate and acids other than carbon dioxide are present. The method of McKinney & Amorosi (1944) corrects for the presence of most interfering substances other than volatile acids, but the titration is performed in open vessels with the consequent loss of the carbon dioxide.

Our method differs from others which use the vacuum extraction technique in that the carbon dioxide content of the sample is related to the resistance changes of a standard solution of sodium hydroxide after equilibration with the extracted gas. Thus the temperature and barometric corrections needed for the volumetric type of van Slyke apparatus (McClendon, 1917) are not required. In addition, the volumetric and manometric methods both require corrections to compensate for the amount of gas which remains unextracted in the sample and for that which is reabsorbed by the sample during the interval between completing the extraction and noting the pressure or volume.

Corrections for both these factors are not required in our method. In the first place the proportion of unextracted gas remaining in the liquid is a constant (van Slyke & Neill, 1924) provided that the volume of the gas phase determined by the mercury level and that of the sample and acid are both constant. Secondly, significant reabsorption did not occur in our experiments when the extracted gas was transferred to the absorbent as rapidly as possible. This is presumably due to the transfer being made to *J* when the pressure in the system was only about +12 cm. Hg, after which the final stages of transfer to the absorbent were made with the gas in contact with relatively dry mercury. This method differs, therefore, from the method of van Slyke where the extracted gas in contact with the sample is often subjected to considerable positive pressure resulting in reabsorption of about 1·4–1·7% of the carbon dioxide.

Since our calibration procedure gives a good linear relationship between the carbon dioxide content of standard solutions and the final resistance of the absorbent, it is justifiable to neglect the corrections for the unextracted portion of the gas and the subsequent reabsorption, provided that the standard procedure is always followed.

APPLICATION OF THE METHOD

Determination of (1) total, (2) carbonate CO_2 and (3) dissolved and bicarbonate CO_2 in natural waters.

Samples were collected in 200 ml. glass-stoppered bottles, with a minimum of disturbance from just below the surface of a stagnant pool in a papyrus swamp. Fig. 5 shows the sampling technique. The water is drawn through a series of bottles by suction from a hand-pump designed for use with a Hearson vacuum-embedding oven. To avoid reducing the pressure to the point at which dissolved gases are extracted and appear as bubbles, the drain vessel must be placed at as low a level as possible and pumping must not be too vigorous. The bottles are immediately stoppered, submerged in water and the total carbon dioxide determination is carried out within 30 min. after collection.

The dissolved and bicarbonate CO_2 was removed by vigorous aeration of the sample and the residual carbonate CO_2 was then determined. The dissolved and bicarbonate CO_2 was obtained by difference. Parallel determinations of the 'free' carbon dioxide (dissolved) concentrations were carried out by the method of Moore (1939) which involves the measurement of pH by glass electrode and of alkalinity by titration with standard acid (see Table 3). The samples were taken from the same swamp on three successive dates and show the progressive effect of flooding during a rainy period after drought. It will be noted that the initial concentration of dissolved and bicarbonate CO_2 was extremely high and that Moore's method gave a higher value for dissolved carbon dioxide. We would attribute this to the

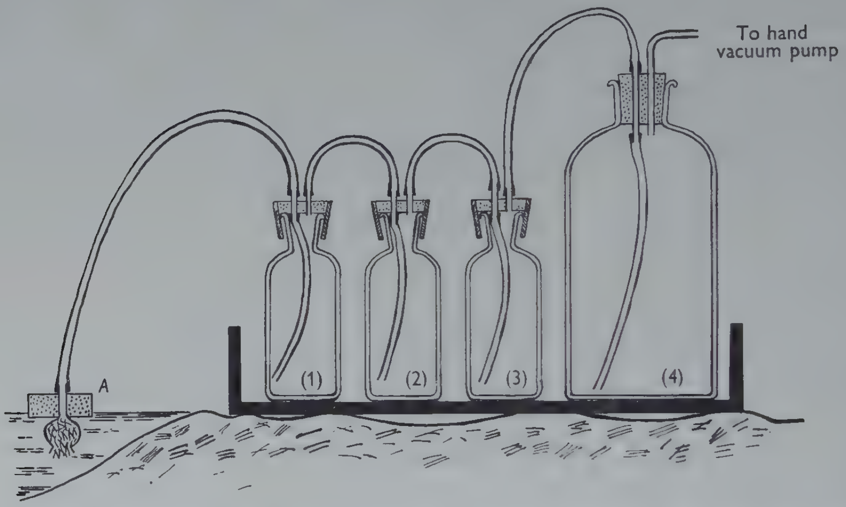

Fig. 5. Method for obtaining samples from a swamp pool. *A*, cork float with tube and glass wool filter. Bottles for: (1) total CO_2; (2) carbonate CO_2; (3) oxygen; (4) drainage.

Table 3. *Carbon dioxide content of water samples from a papyrus swamp, determined by our method and Moore's method* (*Moore*, 1939)

	Our method			Moore's method			
Date	mg./l. total CO_2	mg./l. carbonate CO_2	(A) mg./l. dissolved and bicarbonate CO_2 (by difference)	pH sample	Alkalinity	(B) mg./l. 'free' CO_2 (dissolved)	Difference (A−B) (mg./l.)
1 24. vii. 59	180	32	148	5·9	0·00190N	187	−39
2 21. viii. 59	139	33	106	5·9	0·00130N	127	−21
3 27. viii. 59	71	36	35	6·0	0·00071N	55	−20

presence of non-volatile acids other than carbonic. In one lakeside swamp we found 9·5 mg./l. dissolved and bicarbonate CO_2 by this and 60 mg./l. dissolved CO_2 ('free') by Moore's method. It must, however, be realized that an error of 0·05 in determining the pH is equivalent to 10–15 mg./l. 'free' carbon dioxide with water of this order of alkalinity and pH.

The figures in Table 3 are only quoted to illustrate the use of the method, and cannot be taken as accurately representing the two fractions of these particular samples. It will be seen that the apparent carbonate CO_2 rises slightly in spite of the lower alkalinity values for samples 2 and 3. This was no doubt due to shortening the time of aeration from about 22 hr. (sample 1) to 3–5 hr. for samples 2 and 3. It is clear that the dissolved and bicarbonate CO_2 would be more effectively removed by boiling.

SUMMARY

1. The method described involves extraction of the dissolved gas from an acidified sample in a modified van Slyke apparatus, and measurement of the electrical resistance of a standard sodium hydroxide solution before and after equilibration with the extracted gas.

2. The original sample must be not less than about 30 ml. for which a 10 ml. sample is drawn into the extraction apparatus.

3. There is a linear relation between the resistance finally measured and the total carbon dioxide content of the original sample up to a maximum determined by the strength of the standard hydroxide solution, which is thus adjusted to the desired range of carbon dioxide concentration, e.g. 0·005 N-NaOH for 0–50 mg./l. and 0·02 N for 0–200 mg./l. CO_2.

4. The method is easily sensitive to 0·3 mg./l. in the range 0–50 mg./l. total carbon dioxide and about 1 mg./l. in the range 0–200 mg./l. With a rigidly standardized procedure increased accuracy was obtained so that replicate determinations on standard solutions in the 0–50 mg./l. range did not differ by more than 0·16 mg./l.

5. Precautions for handling samples containing carbon dioxide in solution prior to determination have been suggested.

6. About 15–20 min. are required for each determination and to Mrs D. E.

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FORCE EXERTED BY A SINGLE CILIUM OF *MYTILUS EDULIS*. I

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(With Plate 9)

An important step toward the understanding of the beating mechanism in ciliary movement would be a quantitative analysis of the mechanical properties of the cilium. As a matter of fact, several measurements have been reported concerning the work done by ciliated tissues (Maxwell, 1905) or the force exerted by the whole organism in ciliate protozoa (Jensen, 1893) and flagellated bacteria (von Angerer, 1919). The present paper will deal with the force which a single molluscan cilium can produce during its effective stroke.

MATERIAL AND METHODS

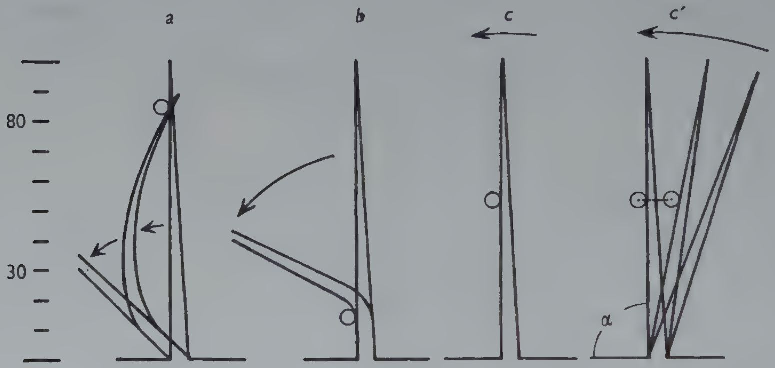
The material used was the large abfrontal gill cilia of the marine bivalve, *Mytilus edulis*. As is clear in Gray's micrographs (1930, 1931), cilia of this kind are convenient on account of their extremely large size (about 50 μ) and sparse distribution; because of the latter condition, the effects of neighbouring large cilia can be neglected. However, surrounding the base of the abfrontal cilia, there are innumerable small cilia covering the entire abfrontal surface of the gill, the influence of which will be dealt with separately later. One large abfrontal cilium consists of several fine subunits which are usually packed together, beating in unison so that the cilium might more appropriately be called a cirrus. Although the subunits can beat independently of each other when they are separated, such separated subunits were excluded from the present experiments.

Kinosita & Kamada (1939), using a glass micro-needle to arrest the movements of single abfrontal cilia, made the following observations.

(1) When a needle is applied at a point near the tip of the cilium (i.e. at a point more than 80% of the distance from base to tip, Text-fig. 1, *a*), the cilium passes under the needle by elastic backward bending and can complete the effective stroke.

(2) When a needle is applied at the proximal part of the cilium, the distal part of the beating cilium bends over the needle and the effective stroke is again completed (Text-fig. 1, *b*). Kinosita & Kamada called such a region which could bend over the needle to complete the stroke the 'active region' and estimated its length as 10–20% of the length of the cilium from base to tip; the present investigation indicates that the active region occupies about 30% of the length of the cilium from base to tip.

(3) If the needle is applied to the mid-part of the cilium (between 30 and 80% of the length) the cilium is arrested without further bending (Text-fig. 1, *c*) and remains motionless until the recovery stroke makes it reverse its motion. Usually the shape of the arrested cilium is straight, as in Pl. 9, fig. 1*a*, although sometimes the proximal part is slightly curved backward (Pl. 9, fig. 1*b*).



Text-fig. 1. Diagrammatic representation of three types of the ciliary responses when a glass needle is applied during the effective stroke. The needle is applied at distal part (*a*), at proximal part (*b*), and at mid-part (*c*, *c′*) of the cilium. Arrows indicate the direction of the effective stroke.

Now taking advantage of situation (3)—motion arrested when cilium is held by mid-region—the force exerted by the cilium can be measured using a flexible needle whose bending characteristic has previously been calibrated (Text-fig. 1, *c′*) This is the principle followed in the present investigation. It is, however, admitted that the application of the method is restricted to the mid-region of the cilium.

Calibration of the bending balance. In a word, the bending characteristic of the micro-needle is calibrated against an actual weight. However, since it is impossible to put a small enough weight on a fine needle whose bending characteristic matches that of the cilium, calibration is done indirectly in several steps by employing a set of needles which differ progressively in compliance. Although this method is tedious, it enables us to determine the absolute value of the compliance of the needles as well as the range of maximum possible error. The procedure for calibration is as follows:

First, several horse-shoe-shaped pieces of copper wire are prepared, and their weights (1–3 mg.) are calculated from their length (1–4 mm.) on the basis of the weight of a known length of the wire (37·8 mg./50 cm.). These are the calibrating weights (error within 0·6%).

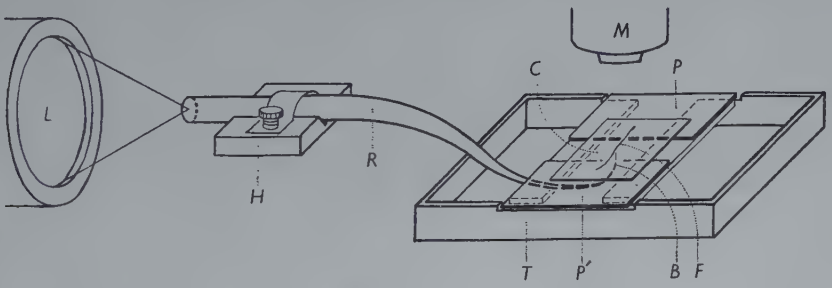
Secondly, three needles which are more rigid than the micro-needles to be used as bending balances are bent by putting the calibrating weights on their tips. The bending characteristics of the needles are determined from the movement of their tips. This first lot of needles will be called 'reference needles' (error within 2%).

Thirdly, many needles, whose compliance varies between that of the reference needles and that of the bending balances, are prepared and calibrated by making them push against each other and against the reference needles in various combinations. In this way the bending characteristics of all the needles can be found (error of this matching process within 15%).

Lastly, three of the needles, calibrated as above, are matched against the bending balances which will be used to arrest the cilia (error within 2·5%).

The range of strengths of the bending balances used is $2{\cdot}5\text{–}6{\cdot}0 \times 10^{-5}$ dynes per 1 μ displacement, the error for this figure being ±16% ($\sqrt{[0{\cdot}6^2+2{\cdot}0^2+15{\cdot}0^2+2{\cdot}5^2]} = 15{\cdot}4$). Deviation from Hooke's Law is less than 2% for a movement of 100 μ, and the mechanical asymmetry around the needle axis is less than 2·5%; both being well within the error of calibration.

Experimental set-up. As shown in Text-fig. 2, a gill filament (*F*) is laid across a slit of 5 mm. between two thin glass plates (*P*, *P′*). The filament is fastened in position by placing a coverglass (*C*) over the slit. This whole assemblage is fitted in a trough (*T*) in such a way that the cover glass is level with the sea water filling the trough. Although placing the filament on its flat side usually brings the beating plane of the abfrontal cilium to a horizontal direction (Gray, 1930), a careful selection is made of a cilium which is beating strictly in a focal plane.



Text-fig. 2. Set-up for dark field recording. *B*, Tip of the bending balance; *C*, coverslip; *F*, gill filament; *H*, holder of micromanipulator; *L*, light source for illuminating the rod (*R*); *M*, microscope objective; *P*, *P′*, pair of glass plates; *R*, glass rod bearing the bending balance (*B*); *T*, glass trough filled with sea water.

A thick glass rod (*R*) (about 10 cm. in length and 7 mm. in diameter) is tapered at one end and fused with a bending balance (*B*) which is bent at right angles at a point about 5 mm. proximal to the tip. The glass rod now bearing the bending balance is so mounted on a manipulator (*H*) as to bring the needle tip upright. By illuminating the basal end of the rod with a microscopic light source (*L*), it is possible to make the tip of the bending balance shine brilliantly as a spot of 1·5–2·5 μ in diameter against the dark background. The movement of the tip caused by the ciliary activity can thus be recorded photographically. In actual records, photographic images of the cilium and that of the loci of the needle tip in the dark field were superimposed by successive exposures on one plate. For the bright field, a bright contrast-phase microscope equipped with a condenser of a long focal length for manipulation was used. Camera lucida drawings which were sometimes made gave essentially the same results as the photographic method.

RESULTS

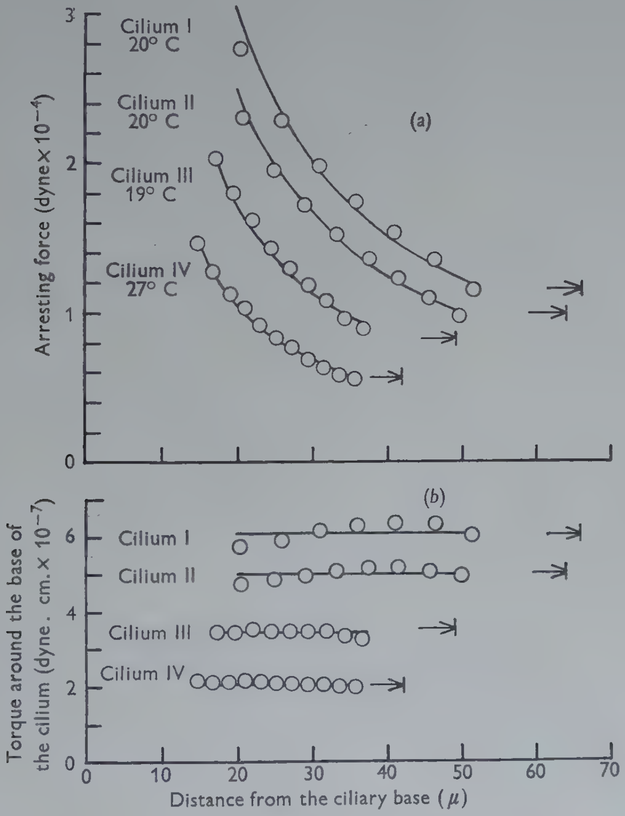
By arresting a cilium at a point between 30 and 80% of its base-tip length, the bending balance is forced to move by several microns and throughout the period of arrest, of from a fraction of a second to several seconds, the cilium and the needle tip are stationary. It is therefore concluded that the cilium continues to exert a constant force on the needle tip throughout the period of arrest and that this force is equal and opposite to the force resulting from the displacement of the needle tip. Such a force will be referred to as 'arresting force'. Occasionally a slight curving of the cilium itself (Pl. 9, fig. 1, *b*) was noticed which, however, was considered to be a passive elastic bending.

In order to make measurements, a photographic film is first exposed to obtain images of a cilium and the gill surface (see Pl. , fig. 2, cilium). Secondly, the field is darkened and the shining tip of the bending balance is applied close to the base of the cilium (about 15 μ distant from the base, *A* in Pl. 9, fig. 2). The spot of light is now oscillating between the arrested (*A'*) and the free (*A*) positions as the cilium moves. The shutter of the camera is then opened and the needle is slowly pulled toward the distal end of the cilium along the straight line previously set (*A–C* in Pl. 9, fig. 2). As the bending balance is pulled, the cilium beats against it and intermittently pushes the needle sideways till equilibrium is reached. The light track on the film shows the successive loci of the needle tip, and consequently the arresting forces at various levels, provided the base-line is given. When the needle is pulled clear of the tip of the cilium (*B*), the light track becomes straight (*B–C*). The preparation is then removed entirely and the needle is moved back along the same straight line once again to obtain the base-line (*C–D*). If the new straight line (*C–D*) failed to coincide with the former line (*B–C*), which sometimes did happen owing to the presence of mucus and other obstacles, the data were discarded.

Text-fig. 3*a* represents four typical results. It is shown that the arresting force decreases as the position of the arresting needle shifts towards the tip of the cilium.

One point in connexion with this procedure requires serious consideration. As was just shown, the magnitude of force which a single cilium can produce varies from one end of the cilium to the other. Consequently, if a bending balance is applied at various levels of a cilium by simply sliding the needle along the cilium, the equilibrium positions of the cilium at various levels change in proportion to the forces exerted, and thus the basal angle α (see Text-fig. 1, *c'*) of the arrested position varies each time. Since it is desirable to unify the data in relation to a more or less constant value of α, the direction of the sliding motion of the needle necessary to stop the cilium within the 10° of the upright position has been previously found empirically. Such line slants toward the end of the effective stroke (line *C–D* in Pl. 9, fig. 2). Although this procedure may seem rather arbitrary in a physical sense, there is a possibility that larger biological complications may be involved if α is not kept constant.

In the introduction the active region of the cilium which is able to bend over the needle was mentioned. However, since the large abfrontal cilium remains nearly straight throughout the effective stroke (Gray, 1930, 1931), under the conditions of these measurements the situation can be looked upon, in simplified form, as follows; the movement of a cilium is due to a bending motion of the cilium restricted to its extreme base on the level of the gill surface. When the cilium is arrested, the force exerted by the cilium is also generated at this basal plane and is in equilibrium with the restoring force of the bent needle by way of an inert straight shaft of the cilium. If this simplified scheme is allowed, the forces which are being generated at the base give rise to a torque, whose magnitude is the product of the arresting force (which is known) and the distance between the level of arrest and the base (which can be measured).

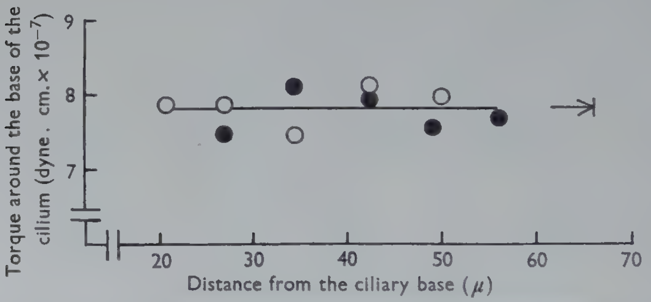


Text-fig. 3. (*a*) The arresting forces at various levels of the cilia. Arrows indicate the total length of individual cilia. (*b*) The torque around the ciliary base calculated from (*a*). Note constancy of it independently of the arresting levels.

This torque, as illustrated in Text-fig. 3*b*, is found to remain constant irrespective of the position of arrest (cilium III and IV). The curves sometimes show a slight upward convexity (cilium I and II), but since this trend falls within the limit of error in taking measurements from the photographic records (±5%, see below), it does not invalidate the statement that the torque is constant irrespective of the position of arrest. It is notable that downward convexity has never been observed.

Table 1

Length of the cilium (μ)	Torque around the base of the cilium (dyne.cm. × 10^{-7})	Temperature (°C.)	Period of a normal beating (sec.)
66	7·8	23	—
66	6·1	20	0·49
64	5·0	20	—
64	3·6	25	0·61
60	3·2	22	—
57	4·2	20	—
53	2·4	22	—
51	3·4	18	—
49	3·4	19	0·50
49	2·7	21	0·57
48	3·7	25	0·52
47	4·3	19	—
46	3·0	19	—
42	2·1	27	0·41
Mean	3·9	—	—



Text-fig. 4. The torque of one cilium measured by two bending balances independently and calculated in the same manner as in Text-fig. 3*b*. Bending characteristics of the balances are $2{\cdot}93 \times 10^{-5}$ dyne/μ (solid circle) and $6{\cdot}03 \times 10^{-5}$ dyne/μ (hollow circle) respectively. Note good coincidence between two series of results. Length of the cilium: 66 μ. Temperature 23° C.

The results are summarized in Table 1. The mean value of the torque is $3{\cdot}9 \times 10^{-7}$ dyne.cm., with some variation ($2\text{–}8 \times 10^{-7}$ dyne.cm.). Measurements of photographic records involve a relative error of 5%. Since the error in the bending characteristic of the balance is 16%, as stated above, the torque has an error of about 16% ($\sqrt{[15{\cdot}4^2+5^2]} = 16{\cdot}1$). In fact, in one case, when the arresting force of the same cilium was measured by two different bending balances, the two series of values coincided very well with each other, as shown in Text-fig. 4.

Effect of small cilia of the abfrontal gill surface

In order to examine the effective range of the stream caused by small cilia, one experiment was carried out in which the tip of a bending balance was gradually brought close to a part of the gill surface where no large cilium existed. It was found that the range where the tip undergoes detectable shift is limited to within 10 μ from the gill surface. It is therefore safe to say that small cilia have no effect upon the values obtained in the present experiments.

Distribution of force-generating capacity within the active region

In the preceding section, the force of the cilium is calculated on the basis of a simplification and expressed in terms of the torque produced by the extreme base of the cilium. But since the proximal 30% of the total ciliary length has the capacity to bend, the active region must be able to produce torque at any level. Theoretically this can be analysed by immobilizing the active region itself at different levels, by applying a rigid needle (as in Text-fig. 1, *b*), and measuring with a bending balance the force which the remaining distal part of the active region exerts. The maximum value of torque obtained when the active region was immobilized at a point 9 μ distal to the base (20% of the ciliary length) was about one-third of the torque obtained at the extreme base. Although technical difficulties made it impossible to inquire further into the activity of the more proximal part of the active region, it can be imagined that the force-generating capacity increases continuously from the distal end of the active region to the extreme base of the cilium.

DISCUSSION

The force which a single cilium can exert has been expressed as a torque acting at the extreme base of the cilium. This is justified in connexion with free ciliary beating because the cilium remains straight all through the effective stroke, the extreme base being the only part which bends.

On the other hand, it has been shown that there is a distal-proximal gradient in the ability of the active region to generate torque. However, from such an observation alone it does not necessarily follow that all the components of the active region are contributing force to the free beating of the cilium. When the cilium is arrested, the active region usually remains straight or, possibly, is curved back as shown in Pl. , fig. 1 *b*. Under this condition, it can also be said that the only part which is actively bending is the extreme base. To say the least, as long as the shape of the active region is maintained constant, the beating force is properly defined as a torque acting at the extreme base of the cilium.

The torque so far defined is that which a cilium can generate when it is arrested during the effective stroke. But whether or not the same torque is generated during movement is a different question. As in the case of isometric and isotonic contraction of muscle, it is very likely that the force developed in free movement would be much smaller and would increase as the viscosity of the medium is raised, reaching a maximum when the cilium is arrested. The author will report on this subject in a subsequent paper.

SUMMARY

1. A bending balance made of a flexible glass micro-needle was prepared, and its bending characteristic was determined by direct matching with calibrated needles.

2. The force produced by a large abfrontal cilium of *Mytilus* was measured by allowing the bending balance to arrest the effective stroke of the cilium.

3. The bending force generated by the cilium is described in terms of a torque, referred to the base of the cilium, amounting to $2\text{–}8 \times 10^{-7}$ dyne.cm. (mean, 4×10^{-7} dyne.cm.).

The author expresses his thanks to Prof. Katsuma Dan for his advice throughout the work and kind help in preparation of the manuscript, and also to Prof. Haruo Kinosita of the Tokyo University for his valuable criticism.

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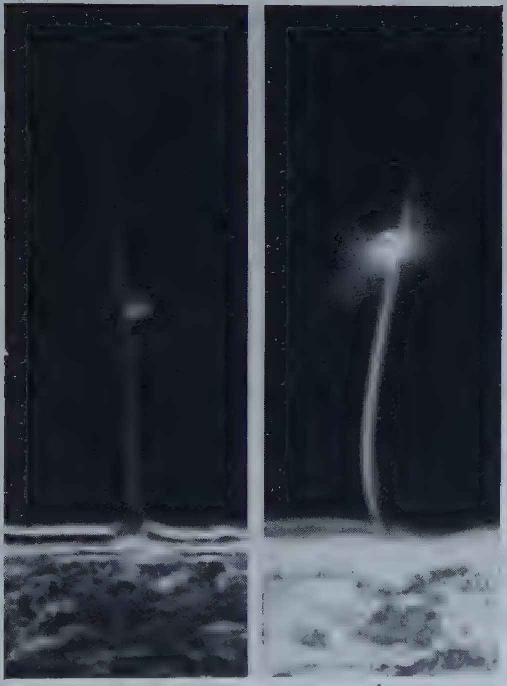
EXPLANATION OF PLATE

Fig. 1. Photomicrographs of the arrested cilia. The shape of the cilium is nearly straight (*a*) or slightly curved (*b*).

Fig. 2. A photographic record. The cilium in this figure lies at the end of the effective stroke. Note gradual decrease in the displacement of the needle tip (therefore decrease in the arresting force) towards the distal end of the cilium. As for the notations, see text. One division of the upper left scale denotes 10 μ.



a *b*

Fig. 1

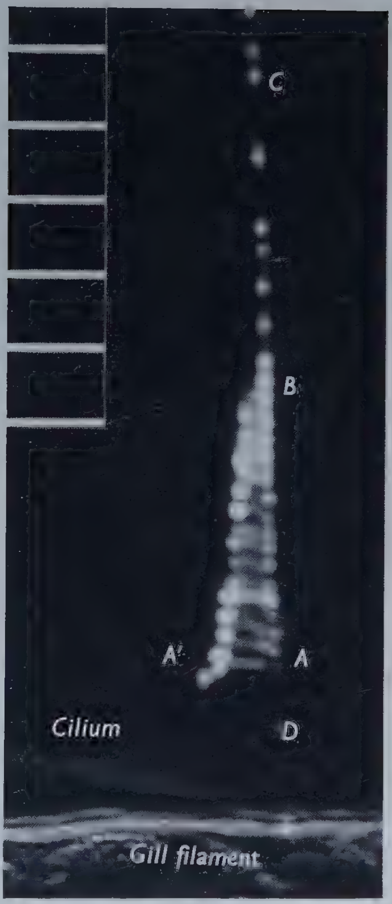


Fig. 2

MITSUKI YONEDA—FORCE EXERTED BY A SINGLE CILIUM OF *MYTILUS EDULIS*. I.

(*Facing p.* 468)

A CINERADIOGRAPHIC STUDY OF THE CENTRAL CIRCULATION IN THE HAGFISH, *MYXINE GLUTINOSA* L.

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(*Received* 21 *January* 1960)

(With Plate 10)

Since Müller (1835) published his pioneer monograph on the morphology of myxinoids, scientists have shown great interest in these phylogenetically important forms. Although works have been presented unabated since then, very few deal with the physiology of these species and almost nothing is known regarding the functioning of their circulatory system.

Retzius (1890) demonstrated the existence of an accessory heart shunted into the venous hepatic circulation and named it the portal heart. He also described and demonstrated the caudal hearts on the venous side in the tail region of *Myxine*. In 1926 Cole demonstrated a similar structure on the deep anterior cardinal vein and termed it the cardinal heart. Recently Johansen (1960), by means of pressure recordings in the vascular system of *Myxine*, presented results that demonstrated an active role of the gills in the propulsion of arterialized blood. Cole (1907) and Hofbauer (1937), among others, have demonstrated histologically the existence of large striated muscle fibres in the gill sacs of this species. Further, the heart of myxinoids is unique among all vertebrate hearts in having no cardioregulatory nerves (Fänge & Østlund, 1954; Greene, 1902; Carlson, 1904; Jensen, 1958). The present study introduces modern radiological methods for the study of the central circulation in *M. glutinosa*.

MATERIAL AND METHODS

The specimens of *M. glutinosa* used for this study were trapped near the Dröbak Biological Station in the Oslofjord in Norway. Only large, adult specimens were used, and they were kept in aquaria with running sea water of 4–7° C. During the experiments the animals were immobilized on a sheet of rubber and immersed in a lucite box filled with cold sea water. The lucite box was easily penetrated by the radiation necessary for the examination. The roentgen equipment consisted of an under-couch tube and a Phillips image-intensifier connected with an Arriflex camera which was operated at a speed of 26 frames per sec. (Johansen & Hol, 1960).

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An incision was made on the ventral side in the posterior part of the animal and the post-cardinal vein was exposed. A polyethylene catheter (P.E. 10) was inserted into the vessel in cephalad direction. Through this catheter the contrast medium was injected under fluoroscopic control. The contrast medium used was 45% Hypaque in doses of around 0·5 c.c. As the animals were surrounded by water their contours were not visible on the films.

OBSERVATIONS

Following a contrast injection, the sinus venosus and atrium became discernible (Pl. 10, fig. 1A). These heart chambers are comparatively large and situated well to the left with the atrium entirely dorsal to the sinus venosus. Thus the pictures of these chambers will overlap each other (Fig. 1A, B) but during contraction of the atrium the sinus venosus is better visualized (Fig. 1B, C). The sinus venosus seemingly consists of two compartments, one anterior and one posterior, both somewhat rounded and separated by a small indentation on the left lateral contour (Fig. 1B). By the force of the contrast injection, and probably also from the contraction of the sinus venosus and atrium, there occurred some filling of the hepatic veins and the jugular or left anterior cardinal veins (Fig. 1A–C). The ventricle is median and slightly to the right and was filled during the contraction of the atrium and sinus venosus (Fig. 1B, C). Neither the sinus venosus nor the atrium showed complete emptying but the residual volumes can be calculated to be less than half of the diastolic contents. The contraction of the ventricle (Fig. 1D) resulted in a considerable but not complete emptying. Following ventricular contraction, the ventral aorta and afferent branchial arteries were filled. The latter may be seen to end at the posterior lateral aspect of the gill bodies (Fig. 1D, E). Heart frequencies varied widely from specimen to specimen with average rate of around 30 beats/min.

This study was primarily initiated because of the evidence from earlier work (Johansen, 1960) that the gills and the muscles in the branchial region take active part in the blood circulation. Outer examination as well as fluoroscopic inspection of the branchial region in *Myxine* revealed that the striated musculature in this region showed repeated contractions. Followed by the high-speed cineradiography, these movements could be resolved and analysed. Fig. 1A, B, shows faintly the gill bodies and the dorsal aorta outlined by contrast from an earlier test injection. The gills are seen oblique and somewhat flattened, corresponding with a contraction of the gill bodies. This position may also be seen directly upon opening of the branchial region. In Fig. 1C, the gills are seen in their early resting phase and the contrast in the dorsal aorta has disappeared. Fig. 2A–D shows the sequence of events during contraction of the gill bodies. In Fig. 2A the gill bodies are relaxed and the contrast uniformly distributed. The dorsal aorta is well defined to the left in the picture. Fig. 2B–D shows progressively the different phases of a contraction. The gill bodies are reduced in size and the contrast in the efferent branchial arteries and the dorsal aorta is squeezed in peripheral direction. Fig. 2E shows the gills relaxed and the dorsal aorta refilled.

Some elucidation of the intricate problem of the existence of extravasation in vertebrates is provided by this study. It seems to be generally accepted that the circulatory system in myxinoids must be considered a semi-closed system, where lacunar spaces (blood sinuses) at some particular sites constitute the means of communication between arteries and veins (Cole, 1926; Marinelli, 1956). Thus the gills in *Myxine* are surrounded by such blood sinuses, the peribranchial sinuses (Cole, 1926). On one occasion we had the opportunity to see the peribranchial sinuses filled with contrast. The afferent path of the contrast medium, injected as usual through the post-cardinal vein, is very difficult to ascertain, but it showed up as a result of overloading the central vessels with contrast. In Fig. 3 the peribranchial sinuses are visible as a number of crescents bordering the gill bodies laterally. Fig. 3 demonstrates the events during contraction of the body-wall muscles in the branchial region. The contraction is followed by a discernible reduction in the amount of contrast in the sinuses. The figure also demonstrates some phases in the muscle contraction. In Fig. 3A, B the contraction starts with a twisting and slight reduction of the diameter. Then follows a shortening and broadening, especially of the distal part (Fig. 3C). The movements end in the resting phase with a stretching and narrowing of the region (Fig. 3D, E).

DISCUSSION

The data presented in this study give evidence to support the hypothesis that the circulatory system in *Myxine* is first of all characterized by a decentralization of the blood-moving power from the heart to the gills and branchial muscles and the earlier-found auxiliary hearts on the venous side of the circulation (Johansen, 1960). Following each gill contraction, blood inside the gill bodies as well as blood in the efferent arteries is expelled peripherally into the dorsal aorta. Further, our results confirm the existence of a direct communication between the vascular vessels and the lacunar spaces in *Myxine* and support our assumption that contraction of the body-wall muscles in the branchial region has an emptying effect on the peribranchial sinuses. Additional information is gained from this study regarding the functioning of the branchial heart *in situ*. It may be emphasized that the heart in *Myxine* possesses a rather high emptying power for all the chambers.

A study using radiological methods was presented by Mott (1950) studying the circulation in the common eel, *Anguilla anguilla*. Contrary to the finding of Mott for *Anguilla*, our study revealed no pulsations in the bulbous aorta of *Myxine*.

SUMMARY

1. An angiocardiographic study has been made of *Myxine glutinosa*, using modern cineradiographic instrumentation. In addition to the heart, vessels in the branchial region have been studied.

2. The topography of the heart chambers and their filling and emptying have been described. The frequency of the heart at body temperature, 8–10° C., was found to be about 30 beats per minute.

3. Results are presented that support the assumption that the gill sacs and their

ducts, as well as striated muscles in the branchial region, take an active part in the propulsion of blood.

4. The phenomenon of extravasation or circulation in lacunar spaces (blood sinuses in direct communication with the true blood-vessels) has been demonstrated. The described muscular activity in the branchial region seems to promote the return of blood from these sinuses to the heart.

Our thanks are due to Prof. Dr Björn Föyn and Cand. Real Finn Walvig for providing the material used in this study.

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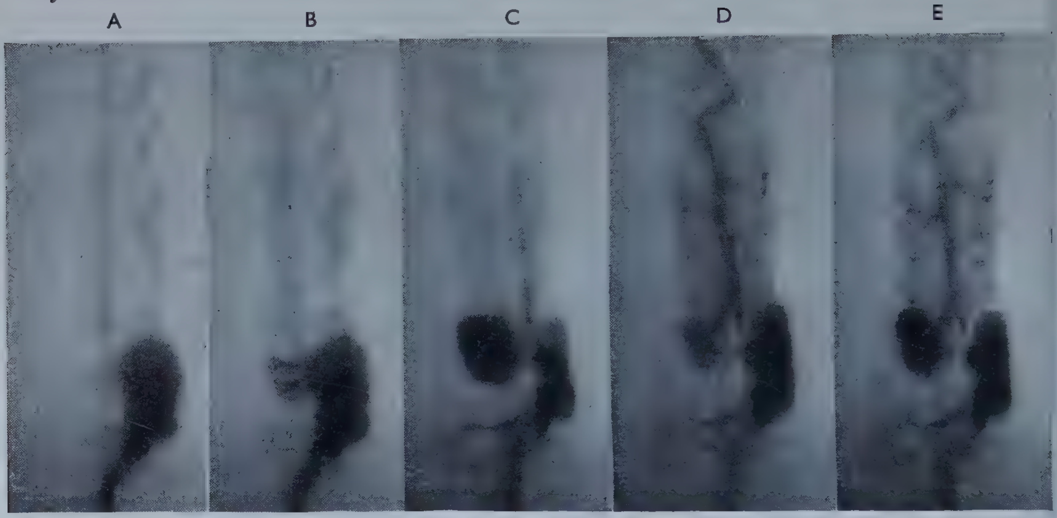

Fig. 1

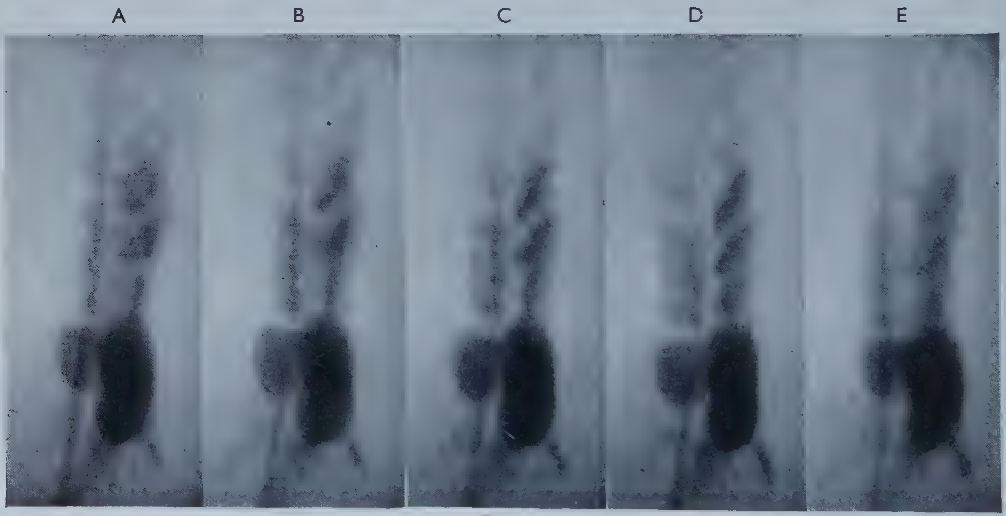

Fig. 2

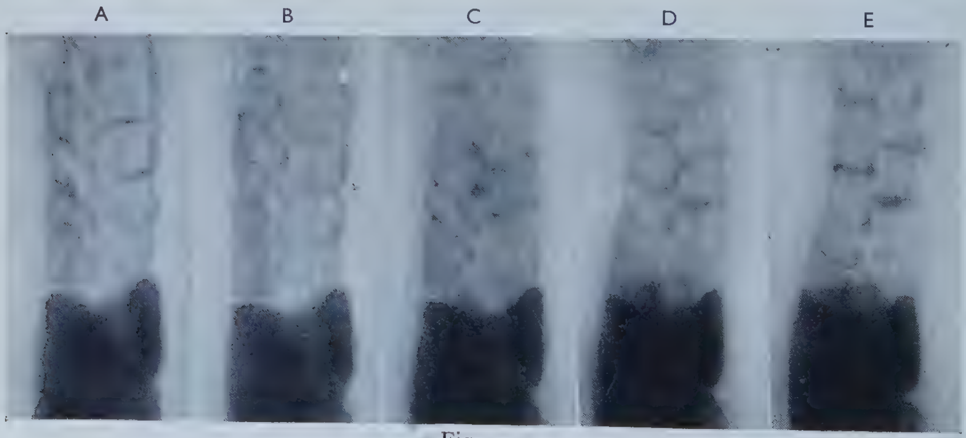

Fig. 3

RAGNOR HOL AND KJELL JOHANSEN—A CINERADIOGRAPHIC STUDY OF THE CENTRAL CIRCULATION IN THE HAGFISH, *MYXINE GLUTINOSA* L.

(*Facing p.* 473)

EXPLANATION OF PLATE

Fig. 1. Angiocardiography of *Myxine glutinosa* (ventral aspect). A. Filling of the sinus venosus and atrium. A few liver branches are seen filled retrogradely. Contrast medium from a test injection faintly outlines the gill bodies and the dorsal aorta running to the right of the atrium. B. Early filling of the ventricle and slight inflow to left jugular and liver veins. Note the left lateral indentation on the sinus venosus. C. The atrium is seen contracted and the ventricle filled; the sinus venosus is also contracted and well outlined. D. Contraction of the ventricle resulting in filling of the ventral aorta and the afferent gill arteries. E. A new atrial contraction fills the ventricle with contrast medium. F. Schematic drawing of the heart and vessels in the branchial region.

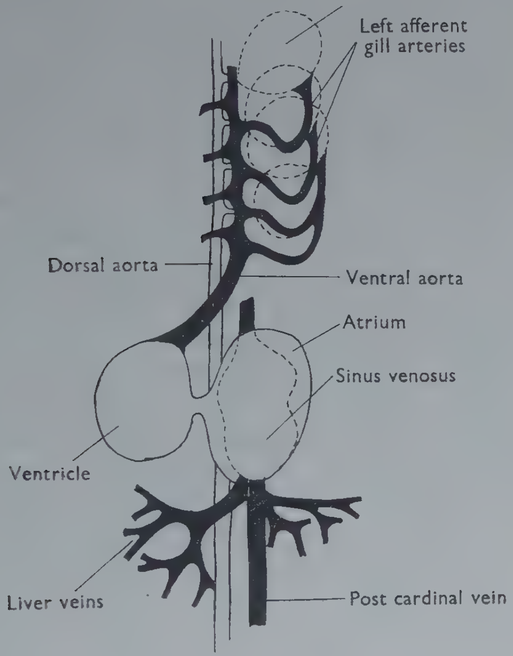

Fig. 1 F

Fig. 2. Gill contraction. A and E. Relaxed gills. B–D. Show progressively different phases of a gill contraction resulting in an emptying of the efferent gill vessels and the dorsal aorta. The amount of contrast medium in the gill bodies is considerably reduced.

Fig. 3. Overloading of the central veins resulting in a filling of the crescent-like blood sinuses bordering the gill bodies. A concomitant change in the shape of the branchial region is seen in connexion with contraction of striated branchial muscles. A. Resting phase. B. Early contraction and some twisting of the gill region. C. Maximal contraction with a broadening. D and E. Early and maximal stretching of the gill region.

A CINERADIOGRAPHIC STUDY OF RESPIRATION IN *MYXINE GLUTINOSA* L.

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(*Received* 21 *January* 1960)

(With Plates 11 and 12)

The anatomy of the respiratory organs, as well as the mechanism of breathing in myxinoids, is distinctly different from that in all other fish-type craniotes even including the petromyzonts. In *Myxine* the branchial gill lamellae are enclosed in rounded sac-like pouches, with ducts conveying the water from the oesophagus to the gills from which it travels through efferent ducts uniting on each side, finally leaving the animal through paired branchial apertures. In addition to the paired gill pouches there exists an oesophago-cutaneous duct connecting the pharynx to the exterior behind the left, last gill sac. The exterior opening is confluent with the left gill pore. Regarding the course of the respiratory current in this system contradictory explanations have been presented. More recent authors seem, however, to agree upon the concept that the water current is solely dependent upon the pulsating activity of the velum and is not influenced actively by either the gill pouches or their ducts (Gustafson, 1935; Strahan, 1958; Marinelli, 1956). The velum and its connected muscles, which is a unique structure in myxinoids, was already anatomically described by Müller in his *Myxine* monograph (1835) and later by Fürbringer (1875) and Cole (1907). The first two authors, however, did not contemplate the velum as being of any functional value for respiration. Cole, on the other hand, suggested a role for this structure in the maintenance of the respiratory current. Goodrich (1930) has advocated that breathing in *Myxine* takes place by expansion and contraction of the muscular elements in the gill sacs. That the gill sacs in *Myxine* contain distinctly striated muscular elements has been described by Cole (1912) and later by Goodrich (1930) and Hofbauer (1937). It seems obvious, upon reviewing the literature, that the physiological interpretations of this problem in earlier works are mostly founded on anatomical studies with little or no physiological experimentation. The excellent works of Gustafson (1935) and Strahan (1958) base their functional interpretations entirely upon external observations of the living animal or upon dropping coloured solution in the respiratory water and following its course into the nostril and out of the branchial apertures. The present study attempts to analyse the mechanics of respiration in *Myxine* by utilizing modern cineradiographic equipment which allows fluoroscopic examination of all events inside the respiratory passages; these may be recorded on a camera at 26 frames per second. The method has been used earlier for the study of the circulation in snakes (Johansen & Hol, 1960) and of the central circulation in *Myxine* (Hol & Johansen, 1960).

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MATERIAL AND METHODS

The specimens of *M. glutinosa* used in this study were trapped near the Dröbak Biological Station in the Oslofjord in Norway. The animals were never anaesthetized but were immobilized in a lucite box filled with cold oxygenated sea water during the experiments. Two different contrast media were used: barium suspension with a particle size of less than 40 μ and the water-soluble Hypaque 45 %. These media were introduced into the animal through a plastic cone fitting into the mouth or a polyethylene tube inserted into the nostril.

RESULTS

Experiments with barium suspension

Most of these experiments were accomplished by introducing the contrast into the pharynx through the mouthpiece. Following contrast filling of pharynx and part of the oesophagus it turned out that no noticeable filling of the gill ducts and gill sacs was achieved. Several different movements could be observed in the branchial region at this time. The afferent gill ducts, scantily filled with barium suspension, could be seen to empty their contents back into the oesophagus (Pl. 11, fig. 1). This could occur simultaneously with increased filling of the oesophagus. By an attempt to overdistend the oesophagus in order to force the barium through the gills, the contrast was ejected forcefully through the oesophago-cutaneous channel (Fig. 2). These experiments are by no means claimed to reproduce normally occurring physiological conditions but were performed in order to elucidate the puzzling problem as to how myxinoids can discriminate the sizes of particles in their respiratory water entering the oesophagus and thereby avoid filling the delicate gill structures with smothering particles.

On one occasion a forceful antiperistaltic movement of the lower oesophagus was observed. By this act both the afferent gill ducts and the lower oesophagus emptied their contents in cephalad direction (Fig. 3). Fig. 4 illustrates another situation during which the oesophagus in the branchial region is contracted, forcing the contrast caudally through the oesophago-cutaneous channel and cranially through the mouth. Fig. 4 also shows a single gill body and its ducts filled with barium suspension which is seen to be squeezed out in peripheral direction. This seemed to be accomplished by contraction of the gill body and its ducts, occurring simultaneously with contraction of the musculature in the oesophagus and the body wall.

Experiments with water-soluble Hypaque 45 %

The water-soluble contrast was injected through a polyethylene tube inserted into the mouth or the nostril of the animal. This contrast medium easily filled the gill ducts and gills without the slightest overdistension of the pharynx and oesophagus. In Fig. 5 the gills and gill ducts are shown well outlined in a somewhat oblique prone position. Following this filling, the various phases of the contrast passage through the gills could be observed. There were no signs of discomfort or unnatural behaviour in the animals during these experiments. The oesophagus and afferent and efferent gill ducts as well as the gills themselves seemed to take active part in the forward propulsion of contrast. In what follows a more detailed description is given as to how each part of the gill system participates.

The afferent gill ducts. During contraction of the gill bodies we observed a narrowing and closing of the afferent gill ducts by sphincter-like mechanisms. The closure took place both at the oesophageal end and at the gill end of the ducts. The former we have named the juxta-oesophageal gill-duct sphincter and the latter the afferent gill-duct sphincter (Fig. 5). The juxta-oesophageal gill-duct sphincter was also seen to contract during the barium injections, when it seemed to be the primary obstacle to the entrance of the suspension (Fig. 2). In the resting phase the juxta-oesophageal gill-duct sphincter was sometimes slightly contracted (Fig. 5 A) while the afferent gill-duct sphincter was open and relaxed. In the early contraction phase the whole branchial region was shortened, most probably brought about by contraction of the m. constr. branch. At this time both the described sphincters were open (Fig. 5 B, C). They contracted and closed the duct lumen during the progressive contraction of the gill bodies (Fig. 5 D) while the juxta-oesophageal sphincter relaxed and opened lumen again during maximal contraction of the gill bodies and the muscles which shorten the branchial region (Fig. 5 E).

The gill bodies. During the initial shortening of the branchial region some further filling of the gills occurred. When the gills started contraction we observed a considerable reduction in their size and they were almost emptied of contrast (Fig. 5 D, E). Often a denser streak of contrast was observed centrally in the gills connecting the afferent and the efferent ducts (Fig. 5 A, D).

The efferent gill ducts. During the described muscular activity definite changes were also observed in the efferent gill ducts close to the exit from the gill bodies. These ducts seemed to be in a slightly contracted state during the resting phase. Immediately before the start of a gill contraction they were wide open, contracting again toward the maximal gill contraction. Thus there exists a third gill-duct sphincter, the efferent gill-duct sphincter (Fig. 5 B, D). The remainder of the efferent gill ducts which unite posteriorly and form a common collecting channel (on the left side also common with the oesophago-cutaneous duct) showed muscular activity of a peristaltic type actively forwarding the respiratory current. This contraction started at the gill end of the ducts and was propagated distally simultaneously with the gill contractions. As previously mentioned, the oesophago-cutaneous channel was observed being used for ejection of superfluous content in the lower oesophagus. These expulsions started and ended very abruptly, pointing to a closure mechanism on this duct also.

Experiments designed to study the activity of the velum

What we could call the upper respiratory tract or the velum region in *Myxine* could also be studied with the technique, although the study of these delicate structures lies on the borderline of what can be well depicted on the films. Through a catheter inserted into the nostril we injected the contrast (water-soluble Hypaque 45%) very slowly. Thereby we achieved a seemingly passive filling of the nasal passages, the velum chamber and the upper pharynx (Fig. 6). Experiments were made both in the antero-posterior and in lateral view. In the former position the movements in the lateral direction were best discernible, while in the lateral view we could better observe the more rapid upward movements of the velum.

The antero-posterior projection. According to Strahan (1958) the velum has a high position in the resting or dead animal. From this position it unrolls ventrally and laterally. By this movement the amount of water in the velum chamber is reduced whereas water flows in between the right and left velar scrolls. These movements were detectable on the films. During the entry of water between the two parts of the velum, the velum chamber and the upper part of the pharynx were slightly reduced in size.

The lateral projection. In the lateral projection the resting phase and the phase of lateral velar movement were difficult to detect. The rapid upward movement of the velum was accompanied by a straightening of the dorsal and ventral contour of the chamber which consequently was reduced in size. Furthermore, the pharynx was wider open. Our observations justify the assumption that the lateral velar movements contribute to the propulsion of water into the oesophagus. On one occasion we followed a combination of movements resulting in a vomiting as shown in Fig. 7. A contraction of the pharynx could be seen to be propagated to the velar chamber and buccal cavity. The position of the velum cannot be distinguished initially but at the end of the contraction the upward maximum or resting position is reached.

DISCUSSION

It is difficult to make definite statements as to the physiological significance of the phenomena described in this paper. The contrast media applied are both foreign and unfamiliar materials to the animals. They have, however, a low toxicity (Hypaque) or none at all (barium). The water-soluble Hypaque was passively injected without causing the slightest discomfort to the animals. There is no doubt that under these circumstances the movements found and described can be easily provoked and give clues to the mechanics of breathing during strictly physiological conditions.

Cunningham (1887) was the first to describe the unusual pattern of respiration in myxinoids, although without giving any explanation as to how the water current is maintained. In 1935, Gustafson made the definite statement that the current of water is driven solely by the velum. Marinelli (1956) confirmed Gustafson's statements and added that the posterior part of the nasopharyngeal duct also pulsates and thus promotes the water current. Recently Strahan (1958), in line with the conclusions of Gustafson, found it untenable that the gill pouches or their ducts participate actively in the propulsion of the respiratory water.

Taking into account the well-developed muscular elements in the gill pouches it seems precipitate definitely to exclude them from an active role in the maintenance of the water current. The results presented in this study leave no doubt that the contractions in the wall of the oesophagus and other branchial muscles, as well as in the gills and gill ducts, may actively assist the velum in the maintenance of the respiratory current. Control of the inflow of water and of its direction may be excited by the system of sphincters in the gill ducts, described above. Our studies also revealed periods when the water flowed rather passively through the relaxed gills but even at this time opening and closing of the gill-duct sphincters was observed, contemporary with a flow of water following central pathways through the gills. When this situation is interrupted by the contraction of the muscular elements in the gill bodies the more tiny water channels inside these gill bodies, constituting the active respiratory area, are inclined to be emptied and refilled, causing an effective exchange of respiratory gases.

Our cineradiographic data from the velum region confirm the statements of Strahan (1958) with one important exception: the velum chamber is not rigid but can reduce its dimensions by contraction. These contractions closely accompany the velar movements and most probably support the backward propulsion of water. We further presume that the phase of lateral velar movements also represents an active stage in the propulsion of water.

An animal like *Myxine* living mostly in muddy regions is permanently exposed to the danger of inhaling particles with a consequent smothering and impairing effect on respiration. According to Strahan (1958) inhalation of large particles evokes a violent expulsion of water through the nostril. This act he has termed sneezing. The mechanism behind this, he states, remains obscure and he suggests as a possible explanation that it resides in the pharyngeal constrictor muscle which encircles the posterior part of the velar chamber. Following contraction of this muscle, water and possibly particles would be driven out through the naso-pharyngeal duct. Occasionally during our experiments we observed this sneezing and recorded it on our films (Fig. 7). The results confirm Strahan's suggestions regarding the influence of the pharyngeal constrictor muscle. In addition, we also noticed contractions of the velar chamber during this sneezing or vomiting. Furthermore, we found it of considerable interest to observe the astonishing ability of the gill-duct sphincters to control the inflow to the gills. The experiments with the barium suspension (particle size less than 40 μ) clearly demonstrated this, and undoubtedly it has great physiological significance to an animal in which the respiratory and alimentary channels have extensive common ducts. The control system to avoid smothering of the delicate respiratory surfaces seems to have a third site of operation in the central channel inside the gill bodies. These channels may represent more open pathways for the water and yield passage to smaller particles which accidentally have passed the afferent ducts.

SUMMARY

1. Cineradiography has been applied to study the respiratory current in *Myxine glutinosa* L.

2. Our observations reveal that contractions in the wall of the oesophagus and other branchial muscles, as well as in the gills and gill ducts, actively assist the velum in the maintenance of the respiratory current.

3. A system of sphincters on the gill ducts has been described. These sphincters function as delicate filters removing particles from the water passing through the gills, and thus preventing smothering of the active respiratory area inside the gill bodies. The gill-duct sphincters take also active part in the directional propulsion of the respiratory current.

4. Additional information has been gained on the function of the velum. Thus the velar chamber is not rigid, but can reduce its dimensions by contraction. These contractions closely accompany the velar movements and probably support the backward propulsion of water. It is further suggested that the lateral velar movements also represent an active stage in the propulsion of water.

Our thanks are due to Prof. Dr Björn Föyn and Cand. Real Finn Walvig for providing the material used in this study.

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EXPLANATION OF PLATES

Plate 11

Fig. 1. A. Afferent gill ducts partly filled with barium from the distended oesophagus. B. The barium contrast is seen rejected from the gill ducts. Time between A and B, $\frac{10}{26}$ sec.

Fig. 2. A, B. Progressive ejection of barium contrast through the oesophago-cutaneous channel. Time between A and B, $\frac{8}{26}$ sec.

Fig. 3. A–C. Antiperistalsis in the lower oesophagus together with rejection of contrast from the gill ducts.

Fig. 4. A. The gills are seen partly filled after overloading and distension of the oesophagus. B. Maximal contraction of the gill region is seen to empty both the lower oesophagus and the gill systems.

Plate 12

Fig. 5. The gill system studied by injection of Hypaque 45 % in water solution. A. Resting phase. The juxta-oesophageal sphincter is partly closed. B. The juxta-oesophageal sphincter opened. C. The efferent gill-duct sphincter opened. Slight twisting of the animal. D. Gill region contraction. The juxta-oesophageal sphincter and the afferent and efferent gill-duct sphincters are seen contracted. The whole gill region is shortened and the gill bodies are contracted with a consequent emptying. There is a noticeable reduction in the size of the gill bodies. E. Maximal contraction of the gill bodies with further emptying. The juxta-oesophageal sphincter is opened again. Time between C and E, $\frac{18}{26}$ sec. F. Schematic drawing of the gill system in *Myxine glutinosa.*

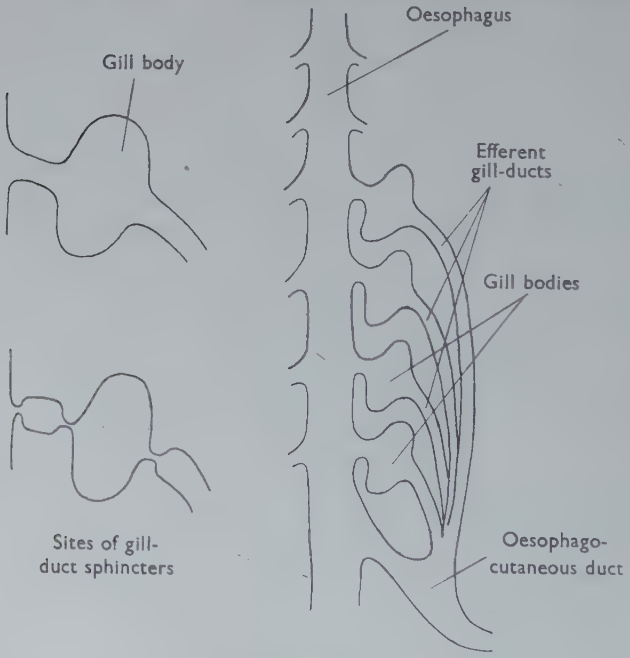

Fig. 5 F

Fig. 6. Filling of the velum region of *Myxine glutinosa.* The contrast medium is injected through a catheter inserted into the nostril. Antero-posterior aspect. A. Good filling of the nasopharyngeal duct and the left part of the velar chamber, early filling of the right part. B. Complete filling of the velar chamber. C, D. Filling of pharynx and oesophagus.

Fig. 7. Sneezing or 'vomiting' in *Myxine glutinosa.* Lateral view. A progressive contraction of the pharynx, velar chamber and buccal cavity drive out the contrast medium through the mouth. A minor amount is expelled through the nostril.

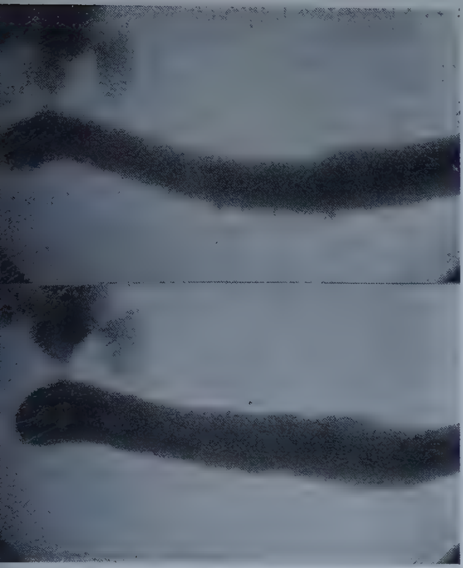

Fig. 1

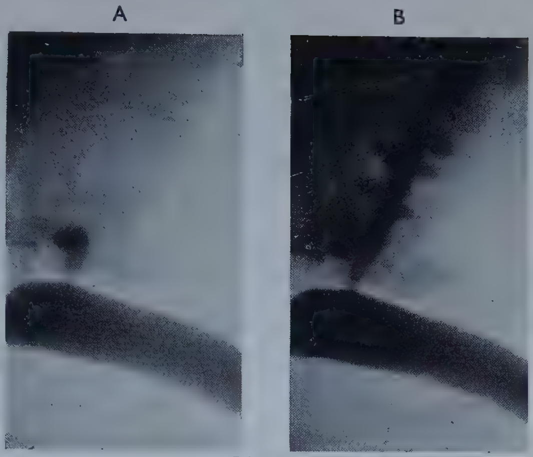

Fig. 2

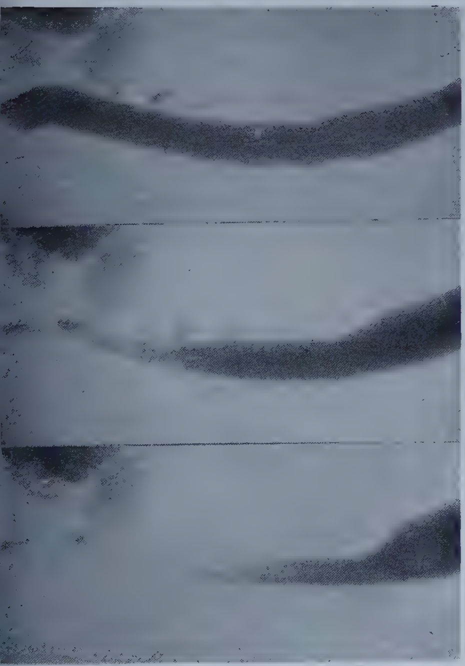

Fig. 3

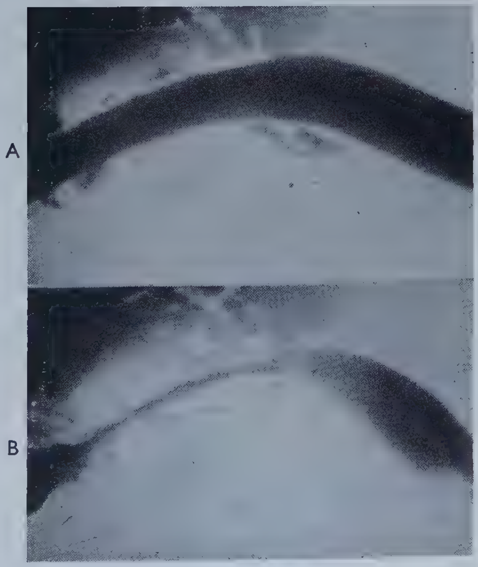

Fig. 4

KJELL JOHANSEN AND RAGNAR HOL—A CINERADIOGRAPHIC STUDY OF RESPIRATION IN *MYXINE GLUTINOSA* L.

(*Facing p.* 480)

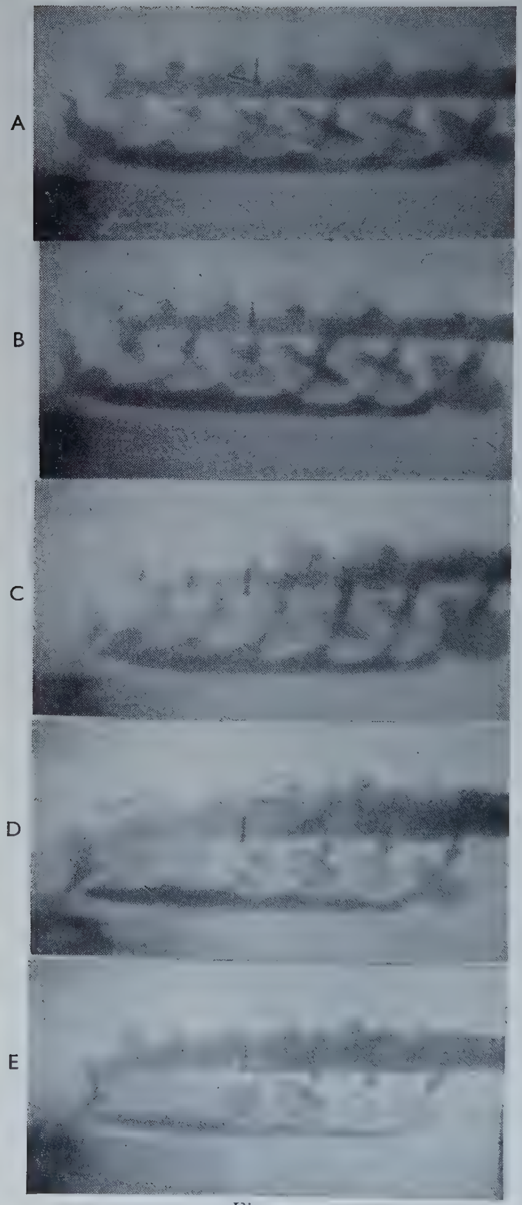

Fig. 5

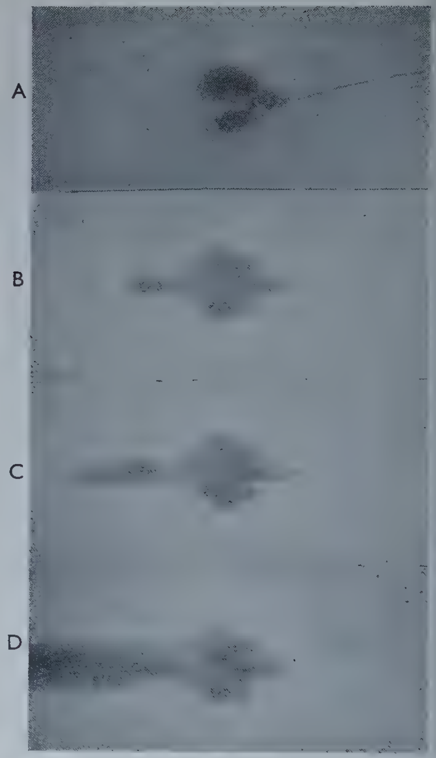

Fig. 6

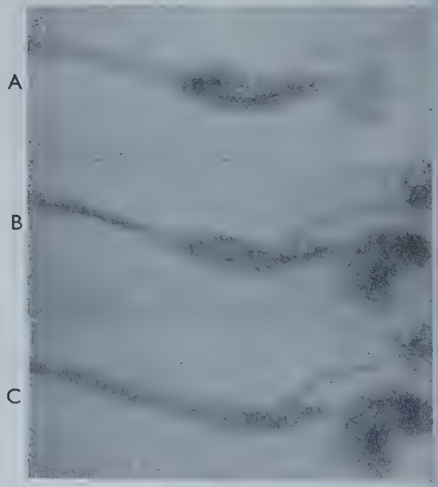

Fig. 7

KJELL JOHANSEN AND RAGNAR HOL—A CINERADIOGRAPHIC STUDY OF RESPIRATION IN *MYXINE GLUTINOSA* L.

LOCOMOTORY RHYTHMS IN *CARCINUS MAENAS* (L.) FROM NON-TIDAL CONDITIONS

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(*Received* 23 *February* 1960)

INTRODUCTION

In a previous communication (Naylor, 1958) it has been shown that specimens of *Carcinus maenas* (L.) collected in the intertidal zone in Britain exhibit a complex rhythm of locomotory activity which persists in constant dim light and at constant temperatures. The rhythm was analysed into two component cycles, one of tidal (12·4 hr.) frequency, and one of daily (24 hr.) frequency. Since then, in an attempt to determine the extent to which the rhythms were 'exogenous' or 'endogenous', it seemed worth while to investigate crabs from conditions in which one of the environmental variables was normally absent or virtually non-existent. In the present experiments, therefore, crabs from a dock in Swansea have been investigated. The daily tidal cycle in the dock is negligible compared with the tidal rise and fall on the open coast, so these crabs ('dock crabs') may be considered to have been subjected to major environmental variables of only 24 hr. frequency and not 12·4 hr. frequency. In addition, crabs collected on the shore ('shore crabs') have been kept in non-tidal aquaria for varying lengths of time in attempts to modify their tidal rhythmicity.

On the basis of these observations, a hypothetical model is suggested to account for persistent rhythms in *Carcinus*. Harker (1958) and Pittendrigh (1958) have recently reviewed work on 24 hr. periodicities and each has suggested a model for an endogenous controlling system, but similar literature on the endogenous control of tidal rhythms is rather less extensive. Brown (1958) considers that the timing mechanisms of daily, tidal and lunar periodicities are largely dependent upon environmental changes, even in so-called constant conditions. The present paper suggests how the tidal and diurnal rhythmicity of *Carcinus*, recorded in conditions of constant dim light and in the absence of tides, may be controlled by internal rhythmic processes.

METHODS

Continuous recordings of the locomotory activity of individual *Carcinus* were made in aktographs constructed of Perspex in which the specimens were kept moist in air (see Naylor, 1958). Activity was measured as the number of tilts recorded by the pivoted aktograph during every hour. As in the previous experiments, the present work was carried out in a room where temperatures varied slightly over a 24 hr. period (± 1° C.) but control experiments were carried out in constant temperature rooms. In most cases the room was darkened and lit continuously by a 25 W. red lamp to offset any differential leakage of daylight. The 24 hr. kymograph drums used with the aktographs were reset at different times of the day to avoid regular periodic disturbance of the animals.

RESULTS

Dock crabs

During the first 24 hr. in continuous dim light in the aktographs dock crabs show predominantly nocturnal outbursts of locomotory activity. Large peaks of activity occur at about 21.00 hr. and 03.00 hr., but there are two much smaller peaks at about 10.00 hr. and 15.00 hr. G.M.T. during what would have been the hours of daylight outside. In successive days, however, the peaks of activity occur progressively later, corresponding peaks occurring about 1 hr. later each day. Such a pattern is discernible for at least 14 days in summated results and is clearly illustrated in Fig. 1, for a period of 6 days. The figure represents the total activity of thirty-two *Carcinus* in five separate experiments, recorded from noon on the first day of each experiment. For reasons which will be apparent later, the grand total is derived by summating the activity assuming that the peaks are delayed by 50 min. each day. The histogram representing the grand total has been drawn at an angle to the remainder to indicate that the final totals are derived in this way. In the grand totals the general pattern of each day's results is thus emphasized to show the two large peaks, which originally occurred at about 21.00 and 03.00 hr. respectively, and the two much smaller peaks which originally occurred during what would have been the hours of daylight outside. These experiments were carried out mainly in spring and late summer when sunrise and sunset occurred at about 05.00 hr. and 19.00 hr. G.M.T., respectively, so the night peaks might be correlated with the times of dawn and dusk. This cannot be a direct correlation, however, for the times of greatest activity do not differ in crabs collected in December when there were 16 hr. between the times of sunset (16.00 hr.) and sunrise (08.00 hr.) (Fig. 2). Moreover, analysis of all the present results shows that the time intervals between activity peaks are the same in crabs maintained at constant temperatures of from 15 to 26° C.

It is evident that the pattern of activity during the first 24 hr. period of any experiment was always the same, which implies that the crabs are predominantly nocturnal in their locomotory habits when in the dock environment. If this is so, then one might expect that the tendency of the peaks to drift would be held in check by a diurnal light cycle. Fig. 3 shows the results of two experiments in which a total of six dock crabs were maintained in aktographs in the normal day/night cycle. The total hourly activity of the six crabs is plotted from 17.00 hr. on the first day of the experiments until noon 7 days later. Apart perhaps from an initial slight tendency to drift, corresponding peaks occurred more or less at the times when they occurred on the first day. The positions of the peaks on day 5 in Fig. 3 approximate closely to those on day 1 in Fig. 1 and are considerably in advance of corresponding peaks on day 5 in Fig. 1. Furthermore, the grand total in Fig. 3 is derived by summating the activity for corresponding hourly periods each day assuming no drift, yet the pattern clearly resembles that in the grand total of Fig. 1. The diurnal cycle has indeed arrested the tendency for the peaks to drift.

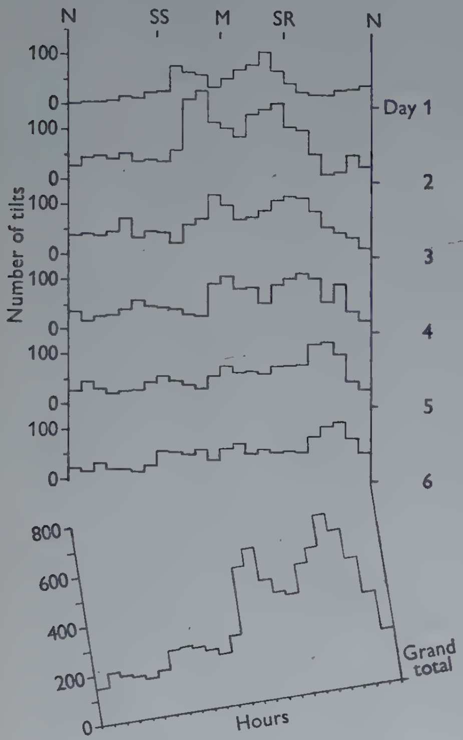

Fig. 1

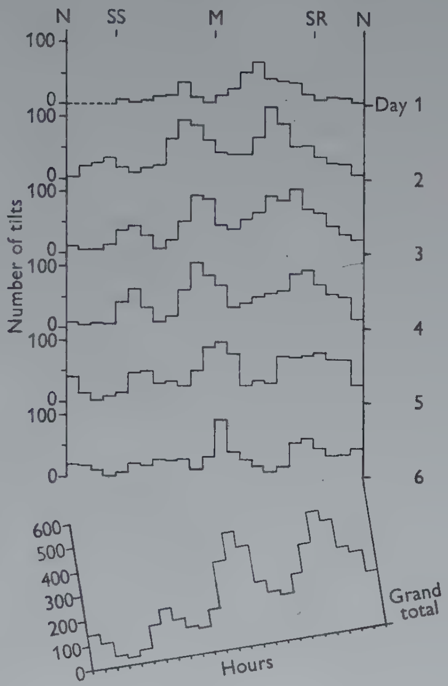

Fig. 2

Fig. 1. Total hourly activity of thirty-two *Carcinus* from a dock during 6 days in continuous dim light (N, noon: M, midnight: SS, approximate time of sunset: SR, approximate time of sunrise). See text for derivation of grand total.

Fig. 2. Total hourly activity of eight *Carcinus* collected from a dock at about the time of the shortest day (December 1959) and kept in continuous dim light (symbols and grand total derived as in Fig. 1).

It is possible that the drift of activity peaks results from the effects of experimental conditions in a way which has been reported for several organisms (see Harker, 1958). This seems unlikely in *Carcinus*, however, since the peaks occur so regularly about 50 min. later in successive days, like the tides and like the activity peaks in crabs collected from the shore (Naylor, 1958), and since the cycles are independent of temperature (see above). Furthermore, in cases where drift results from the nature of the experimental conditions, it has been shown that if darkness consistently delays a rhythm then continuous light advances it, and *vice versa* (Aschoff, 1958). In *Carcinus* there is no evidence of such a reversal. Eight crabs kept in various parts of a continuously lit constant-temperature room at 15° C. in light intensities of from 15 to 75 foot-candles showed activity peaks which occurred progressively later each day. The drift was similar to that exhibited by dock crabs in continuous dim red light at a light intensity of much less than 1 foot-candle. It is perhaps more likely, therefore, that dock crabs, which are presumably of the same stock as crabs from the shore outside the dock, possess a persistent physiological rhythmicity which is of approximately tidal frequency, even though they have lived for some time in non-tidal conditions. It is fairly generally recognized that rhythmicity can be so persistent and could perhaps even be an inherited feature of living organisms (Harker, 1958; Pittendrigh & Bruce, 1957). If rhythms of tidal periodicity are highly persistent, then the occurrence of the two small daytime peaks can be accounted for (see p. 487).

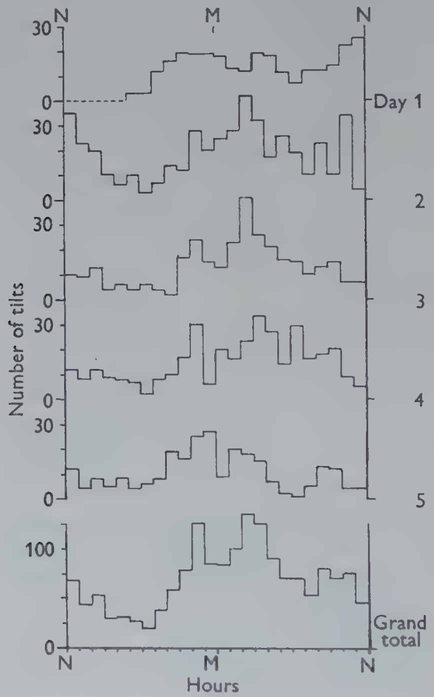

Fig. 3. Total hourly activity of six dock *Carcinus* which were kept in the normal light/dark cycle of day and night (symbols as in Fig. 1).

Shore crabs

In an attempt to modify the tidal rhythmicity of shore crabs, these were collected from Mumbles Point, Swansea, and kept in an aquarium in the normal day/night regime of the laboratory. At intervals individuals were removed to aktographs in continuous dim light for a period of 24 hr. in order to test their rhythmicity, and were then returned to the aquarium. When first collected the crabs showed a characteristic rhythm which was in phase with the tidal cycle on the shore. Thenceforth rhythmicity was less clear-cut, and after a period of about 4 weeks, the shore crabs exhibited a rhythm similar to that of dock crabs. At this stage in the experiments the crabs were kept in constant dim light and activity was recorded for a period of 6 days. Fig. 4 shows the total hourly activity of eight shore crabs, three of which had been in the aquarium for 8 weeks and five of which had been there for 4 weeks. The initial pattern of locomotory activity and the subsequent drifting of the peaks are as observed in dock crabs (see Fig. 1). Thus when shore crabs are transferred to non-tidal conditions, indications are that though the pattern of locomotory activity changes the tendency still persists for peaks of activity to drift at the same rate as the tides.

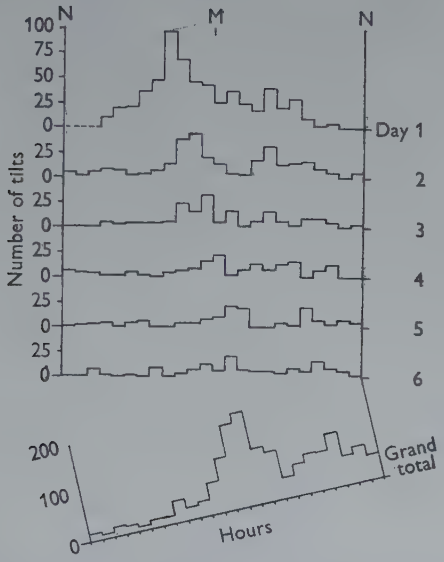

Fig. 4. Total hourly activity in continuous dim light of eight shore *Carcinus* collected 4–8 weeks previously and maintained since collection in a day/night cycle in an aquarium (symbols and grand total derived as in Fig. 1).

A 'MULTIPLE CLOCK' HYPOTHESIS FOR THE CONTROL OF RHYTHMICITY IN *CARCINUS*

This tendency for the peaks to drift, together with the appearance of small daytime activity peaks in dock crabs (see p. 482) is evidence that rhythms of tidal frequency persist in crabs which have been isolated from tidal conditions. In non-tidal conditions such rhythms can be modified or held in check by diurnal light/dark cycles in the environment, but find expression again in rhythms of activity with a tidal frequency when the organism is kept in continuous dim light. The question of how rhythms may persist to such an extent and yet still be modifiable by environmental or experimental stimuli remains to be considered.

Harker (1958) has recently suggested a model to account for the control of 24 hr. periodicities in plants and animals. The model postulates not one 'biological clock' within the organism but many, possibly of a cellular nature. These 'clocks' might be in phase or at variance, determining whether the organism exhibited an overt rhythm or not. Such a model has been used by Cloudsley-Thompson (1958) to account for the endogenous chronometer in *Gryllus campestris*. A similar model may also be used for *Carcinus*, assuming that some of the 'multiple clocks' have a basic periodicity of approximately tidal or 12·4 hr. frequency and that others have a frequency of perhaps twice that value. On this assumption one could postulate an hypothetical, apparently arhythmic, crab in which the rhythmicity of its 'clocks' still persisted. Thus such an organism could possess many cellular rhythms of either frequency, all of which would be operating at variance, and no overt rhythm of locomotory activity would be expressed (Fig. 5*a*). In order to relate the cellular rhythms to the observed locomotory rhythm it is convenient to deal separately with the tidal and diurnal components.

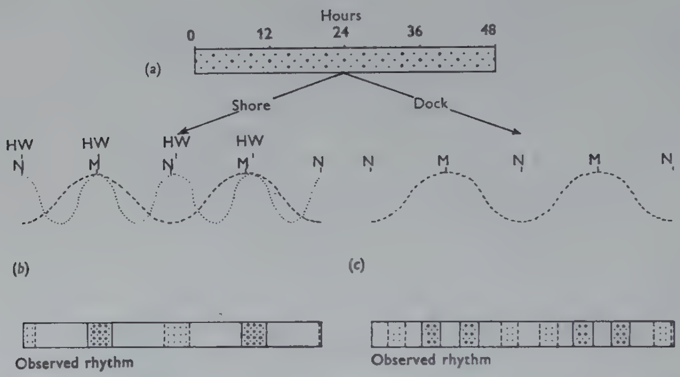

Fig. 5. 'Multiple clock' hypothesis for the control of locomotory rhythms in *Carcinus*; (*a*) 'clocks' at variance and no locomotory rhythm apparent: (*b*) under the influence of environmental cycles of tide and light 'clocks' operating at the time of each high tide and at night: tidal rhythm observed with nocturnal peaks exaggerated (see Naylor, 1958); (*c*) in non-tidal conditions 12·4 hr. 'clocks' operating at night (two peaks) and again in daylight (two smaller peaks) with individual 'clocks' operating at alternate peaks: 24·8 hr. 'clocks' operating to produce the high nocturnal peaks. (◡◠, tidal cycle: ◡◠, diurnal cycle: HW, time of high tide: (●), 24·8 hr. 'clocks': (·), 12·4 hr. 'clocks': other symbols as in Fig. 1).

The tidal component

In a shore crab the 'multiple clocks' would be brought into phase with each other to restrict locomotory activity to those times when it was of survival value for the animal. Shore crabs might be expected to be most active when the tide is in and to seek shelter when the tide is out, as indeed seems to be the case (Naylor, 1958), and any rhythmic process controlling such behaviour might be expected to be phased accordingly. In this way the tidal 'clocks' could be so phased as to operate together every 12·4 hr. and to continue doing so for some time, even when the animal is removed from the shore (Fig. 5*b*).

The length of time for which the rhythms would persist would clearly depend upon the frequency of the 'multiple clocks'. If the frequency of each 'clock' was *exactly* tidal (or diurnal), then the cellular rhythms could not drift out of phase in the absence of environmental cycles and it would not be possible to modify by experiment the expressed locomotory rhythm. If, on the other hand, individual 'clocks' varied and it was only the mean frequency which was approximately tidal (or diurnal), then in crabs kept in constant conditions, the 'clocks' would drift out of phase and the overt locomotory rhythm would become less precise. Rhythms do, in fact, become less precise in *Carcinus* kept in constant conditions, as they do in other organisms (Harker, 1958).

By means of such a model it is possible also to explain much of the behaviour of *Carcinus* in non-tidal conditions. The results presented here show that crabs, kept continuously immersed in sea water in the normal day/night cycle, become active mainly at night, with greatest activity occurring in the late evening and in the early morning. It is not clear why there are two nocturnal peaks, but one might expect some of the 'multiple clocks' to be phased together at each of these times. Since the two nocturnal peaks are only about 6 hr. apart (see p. 482), it is necessary to postulate that different 'clocks' are involved in each nocturnal peak of locomotory activity. If different 'clocks' are involved, then the presence of the two smaller peaks during the hours of daylight (Fig. 1) may be explained. Each daytime peak occurs about 12–13 hr. after a corresponding nocturnal peak. It seems that one group of tidal 'clocks' is phased to operate just before midnight and again about 12·4 hr. later, just before noon, whilst another group is phased to operate at about 03.00 hr. and again about 12·4 hr. later, in the late afternoon (Fig. 5*c*).

The diurnal component

A persistent diurnal component is apparent in the rhythm of both dock crabs and shore crabs. High nocturnal peaks alternate with low daytime peaks when animals from each habitat are kept in continuous dim light. These exaggerated outbursts of activity could be accounted for if cellular 'clocks' of diurnal frequency were in phase with those of tidal frequency at night time. Each diurnal 'Clock' would then operate again about 24 hr. later, by which time the tidal 'Clocks' would be operating again for the second time (see Figs. 5*b* and 5*c*). However, it is clear that in shore crabs and in dock crabs the high nocturnal peaks drift at the same rate as the tides when the animals are kept in continuous dim light. So, either the drift is a direct response to the experimental conditions, which seems unlikely, or the diurnal 'clocks' are of more nearly twice tidal frequency (24·8 hr.) than diurnal frequency (24 hr.). Indeed, in crabs on the shore activity is most pronounced at high tides which occur during the night and it is clear that the exaggerated peaks at those times are consistently about 24·8 hr. apart (Naylor, 1958).

At present, therefore, the most economical hypothesis should perhaps be that there are two types of internal chronometer, one of tidal frequency and one of about twice that value. The periodicity of each type is fairly flexible and it approximates to the frequency of an environmental cycle by which it is normally constantly rephased (see Harker, 1958; Cloudsley-Thompson, 1958).

SUMMARY

1. Locomotor activity has been continuously recorded by aktograph in *Carcinus maenas* (L.), using (*a*) 'dock crabs' from non-tidal docks, and (*b*) 'shore crabs' from the intertidal zone.

2. Dock crabs, kept in continuous light, either dim or bright, show a temperature-independent rhythm. During the first 24 hr. large peaks occur after dusk and just before dawn, with smaller peaks during the day. Each daytime peak occurs about 12–13 hr. later than a night peak. Subsequently all the peaks drift, occurring about 50 min. later each day.

3. Dock crabs kept in a day/night light regime do not show this drift.

4. Shore crabs, after 4–8 weeks in non-tidal aquaria, behave in the same way as dock crabs.

5. It is suggested that the drifting of the peaks represents an expression of endogenous tidal rhythmicity and a 'multiple clock' hypothesis is postulated to account for the control of rhythmicity.

My thanks are due to Prof. E. W. Knight-Jones for much helpful discussion and to Mr A. Milward who constructed the aktographs.

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PROPRIOCEPTION AND VISUAL DISCRIMINATION OF ORIENTATION IN *OCTOPUS*

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INTRODUCTION

Octopuses can be trained to discriminate between figures seen that differ only in orientation. Thus a rectangle shown with its long axis vertical can readily be distinguished from an exactly similar rectangle shown horizontally (Boycott & Young, 1957; Sutherland, 1957). A wide variety of cues are potentially available to octopuses in the training situation used; the figures might, for example, be recognized as distinct because they are parallel or not parallel with the water surface, with the sides of the aquaria, or with the direction of illumination. Since the figures are moved up and down on the ends of rods inserted into the tank, they could also be distinguished as moving along or at right angles to their long axes (see Sutherland & Muntz, 1959) or as parallel or not parallel with the supporting rod. An alternative set of cues is potentially available from within the animal itself, in the form of proprioceptive information about the position of the animal in space, originating in stretch or pressure receptors and/or in the statocysts.

It will be shown below that octopuses cannot reliably discriminate between figures differing only in orientation after removal of the statocysts, and that correct performance in such discriminations depends upon correct orientation of the retina, and not upon central integration of visual and positional information.

MATERIAL AND TRAINING TECHNIQUE

Octopus vulgaris Lamarck. from the bay of Naples was used, experiments being made in July and August 1958 (EK series) and 1959 (FK series). The animals used were all very small compared with those used hitherto in octopus training experiments and weighed between 16 and 36 g. when killed after a period of training during which they grew appreciably in size.

Each individual was kept in a separate tank, either a glass accumulator jar (with black paper screening around it) or (more often, including the whole of the FK series) in a wooden aquarium measuring 40 × 20 × 15 cm. A jet of sea water provided circulation and aeration, and each animal was provided with two or three pieces of brick grouped at one end of the tank to form a 'home' in which it normally sat. Each tank had a lid.

Animals were required to learn to discriminate between the members of pairs of figures, cut out of card, presented successively and moved up and down at the end of the tank distant from the octopus's home. Animals were rewarded with a fragment of sardine or shrimp for attacking one (the 'positive') of the two figures, and were given a 3–5 V. a.c. electric shock when they attacked the other (the 'negative' figure). The experimental situation was thus as nearly as possible a copy of that originally devised by Boycott & Young (1955), which has been used in the majority of subsequent visual training experiments with larger octopuses (for a review of these see Young, 1960*a*). Training was at a rate of ten trials per day, 5 + and 5 −, at intervals of not less than half an hour; the trials were systematized thus: + − + − + + − − + −, next day − + − + + − − + − + and so on.

OPERATIVE TECHNIQUE

The principal reason for using small octopuses in these experiments was that it is relatively easy to remove the statocysts from young animals. After anaesthesia in 3% urethane, an octopus of 20 g. or less is sufficiently transparent for the anterior vena cava to be seen pulsating beneath the skin. Avoiding this blood-vessel constitutes the only serious difficulty in removal of the statocysts from below. To remove each statocyst an incision is made in the skin and muscle beside the anterior vena cava to reveal the cartilage surrounding the brain. The white otolith of the statocyst can be seen clearly through the cartilage. (In very small specimens the skin and muscle are transparent too, and the otoliths can be seen before any incision is made.) The statocyst can be removed entire by picking out the endolymphatic sac with fine forceps through a hole made in the cartilage. The whole operation is very rapidly done, and if the initial incision is made parallel with the muscle fibres immediately underlying the skin, the wound heals quickly and completely. One week after operation the lesion is only detectable because the chromatophores in the skin overlying the wound tend to be more expanded than their immediate neighbours. The effect of the operation upon the behaviour of the animal is so striking and regular that a subsequent histological check upon the completeness of the operation was not considered necessary.

IMMEDIATE EFFECTS OF STATOCYST REMOVAL UPON MOVEMENT AND POSTURE

Boycott (1960) and Dijkgraaf (1960) have described the effects of unilateral and bilateral statocyst removal upon the movements and posture of *Octopus*. The animals used by them were all much larger than those used in the present series of experiments, but their behaviour after operation was sufficiently similar for there to be no point in redescribing it in full here. Briefly, removal of a single statocyst, left or right, produces no detectable changes in behaviour; but removal of both statocysts causes widespread abnormalities of movement and posture. Animals with both statocysts removed cannot swim straight and tend to spiral in an irregular manner. They are therefore unable to approach objects seen at a distance in the usual way by swimming up to them, aided by jets from the funnel. Pretrained animals tested after bilateral statocyst removal sometimes try to make such attacks, but rarely reach their objective. Octopuses with both statocysts removed can, however, crawl rapidly about their tanks, but again their progress, particularly when some of the arms are not in contact with the substratum, is erratic. The head tends to jerk from side to side, and the animal is liable to fall over its own arms if moving rapidly. Statocyst removal appears to have no effect upon muscular tone; the operated animals are in no way 'weak' and they climb out of their tanks if anything more frequently than unoperated animals. The main difficulty of training such animals is that though they react readily by starting towards objects seen, their approach path is often so erratic that the target fails to remain within the visual field; an animal that has started to come down the tank frequently stops, apparently unable to see the target until further movement of its own head brings this again into sight. Two changes from the usual visual training technique were made to allow for this difficulty. First, the duration of the individual trials was increased from 20 or 30 sec. to a full minute; this allows time for several 'false starts' and for a slow intermittent approach. Secondly (in the FK series of experiments only), the figures were shown relatively close to the animals instead of at the full length of the tank. In the last 10 days of the experiments with the FK series the figures were shown sufficiently closely for the animals to reach out and take them without alteration of bodily position, so that the position of the eye concerned did not alter appreciably in the course of the response.

EFFECT OF STATOCYST REMOVAL UPON THE ORIENTATION OF THE EYES

One of the most striking effects of bilateral destruction of the statocysts is upon the orientation of the eyeballs. In unoperated octopuses the slit-like pupil of the eye remains horizontal, or nearly so, however the animal is sitting in its tank (Fig. 1 *a*–*e*). After removal of one statocyst this remains true, even of the side of the animal from which the statocyst was removed. After bilateral operations all regularity of orientation is lost. The position of the pupil then depends upon the position of the head, and this in turn depends upon the position in which the animal happens to be sitting (Fig. 1 *f*, *g*).

It will be shown below that figures differing only in orientation are distinguished by their positions relative to the retina, as indicated by the pupil, and that animals deprived of their statocysts after training make mistakes in such discriminations when their pupils are in an abnormal (i.e. vertical rather than horizontal) position.

EXPERIMENTAL RESULTS

Animals were trained to make either a black-white or an orientation discrimination. The black-white discrimination was between two circular figures each 12 mm. in diameter, the other was between 5 × 30 mm. rectangles shown with the long axis vertical or horizontal. All the animals were pretrained to attack the future positive of the two figures to be discriminated before the start of the discrimination experiments.

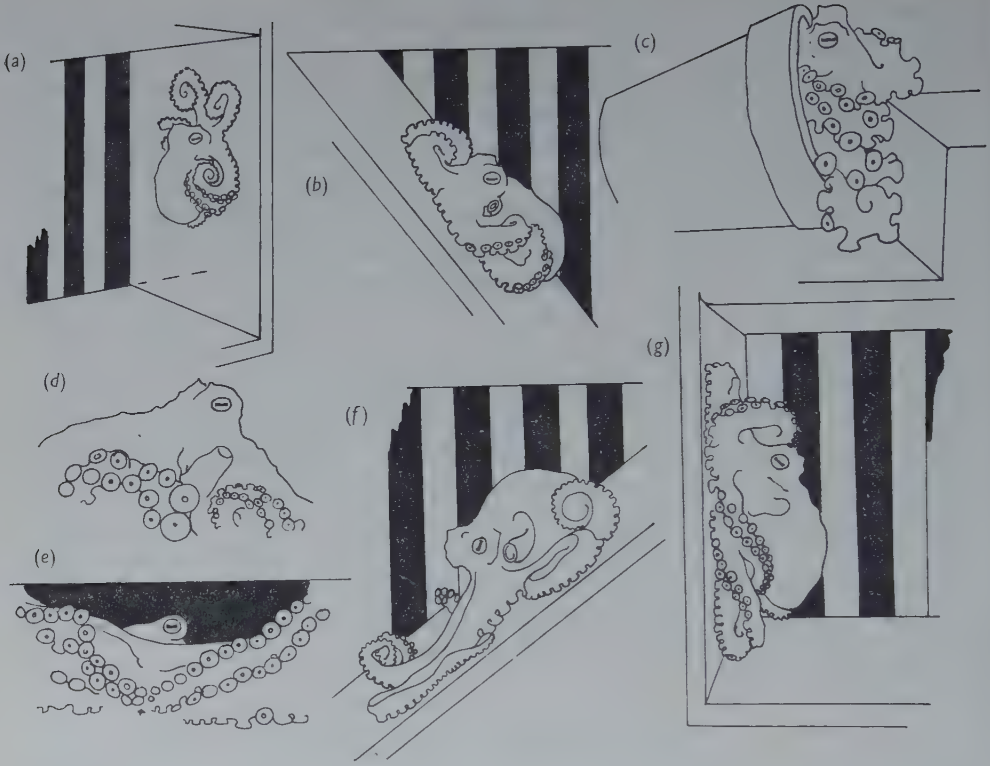

Fig. 1. Orientation of the eyes before and after bilateral statocyst removal. In unoperated animals the slit-like pupil normally remains horizontal or very nearly so (*a–e*), whatever the position of the octopus. After removal of both statocysts this ceases to be true, and the orientation of the retina, as indicated by the position of the pupil, thereafter depends upon the position in which the animal is sitting (*f–g*, octopus EKK). Pictures traced from projections of Kodachrome transparencies: *c*, *d* and *e* are of comparatively large (500 g.) octopuses, the rest of small (15–25 g.) animals in an aquarium set up in front of a vertically striped background; in *b* and *f* the aquarium, with the animals sitting on the bottom, has been tipped through 45°.

In the orientation discrimination experiments nine of the animals were trained with the vertical, and six with the horizontal rectangle as the positive figure; in the black-white discriminations four animals were trained with the white and three with the black circle as positive figure. Young (1958) has shown that untrained, though somewhat larger (500 g. ±), octopuses are slightly more likely to attack vertical than horizontal figures and white rather than black figures under the experimental conditions used here. Details of the direction of training in individual experiments are given in Tables 1 and 2, and in Fig. 3.

Three of the octopuses tested had both statocysts removed before training; the rest, nineteen in all, were pretrained to discriminate before operations in which one or both statocysts were removed. The duration of pretraining varied considerably, this being continued until the animal concerned was regularly making better than 75% correct responses; the octopus was then operated at the end of a day's set of ten trials, training being resumed next morning. Fig. 2 summarizes the results of experiments with pretrained animals. In it only the results of the last thirty preoperational and the first thirty or fifty postoperational training trials are given, although most of the animals were trained for much longer. Details of some individual performances are given in Fig. 3.

Table 1. *Details of attacks made after removal of both statocysts*

Figures to be discriminated	● ○ (B) *v* (W)					▯ ▭ (V) *v* (H)		
	Attacks on					Attacks on		
Positive figure	+ve	−ve	Total trials		Positive figure	+ve	−ve	Total trials
			Pretained animals					
FK 1 B	62** (25)	18** (11)	140		FK 7 V	12 (1)	17 (4)	180
FK 3 W	26** (12)	6** (4)	100		FK 8 V	47* (9)	21* (4)	180
FK 6 W	61** (22)	16** (9)	130		FK 9 V	3 (2)	8 (7)	80
EKR B	13 (13)	5 (5)	50		FK 10 V	42* (9)	21* (5)	180
EKS B	18* (18)	5* (5)	50		EKF H	12 (4)	9 (4)	90
					EKH H	6 (5)	6 (5)	60
					EKI H	16 (13)	8 (8)	70
					EKJ H	1 (1)	1 (1)	50
					EKL H	6 (6)	4 (4)	70
					EKM H	5 (4)	9 (7)	70
			Animals without preoperational training					
FK 11 W	29**	7**	130		EK 6 V	29	19	140
FK 12 W	39**	9**	130					

** Difference significant at the 0·1 % level.
* Difference significant at the 1 % level.
Figures in parentheses show the number of attacks made in the first fifty trials after operation (as summarized in Fig. 2*b* and *c*).

It can be seen (Fig. 2) that removal of one statocyst did not affect the performance of octopuses in the orientation discrimination (8 animals). Removal of both statocysts, on the other hand, abolished the capacity to discriminate between the rectangles (10 animals), while not preventing discrimination of black and white circles (5 animals). The performance of three further octopuses from which the statocysts were removed *before* training confirms this result; the two trained on a black-white discrimination learned, while a third animal, trained using the rectangles, did not make significantly better than chance responses (Table 1).

The apparently simple result that bilateral statocyst removal abolishes the capacity to make orientation discriminations is seen to be more complicated when the performance of individual animals is considered. Individual records show that attacks upon the rectangles were *not* always made at random after operation; certain animals scored significantly better than might be expected on a chance basis, and nearly all showed a bias in favour of one or other of the test objects. The non-discriminatory responses of the group considered as a whole (Fig. 2*b*) are a consequence of summing the performances of individuals most of whom discriminated postoperationally to some extent, though not always in the direction learned during preoperational training; thus in the first fifty postoperational trials three out of ten animals tested actually took the negative object more often than the positive, and three took negative and positive equally often; only four out of the ten octopuses continued to discriminate in the direction learned during pretraining.

These results become explicable when the responses made are considered in relation to the postoperational habits of the individuals concerned. An octopus tends to return to a particular location on the side or bottom of its tank when not wandering about, and this determines the posture in which the individual is usually found when at rest at the start of a training trial. The position that the operated animal takes up in its tank affects, among other things, the orientation of its retina, as indicated by the pupil (Fig. 1*f*, *g*). During the last 10 days of the 1959 (FK) series of experiments shown in Fig. 3 the position of the pupil was recorded together with the record of responses made. It was found that two of the operated octopuses (FK7 and FK9) tended to sit in positions such that their pupils remained

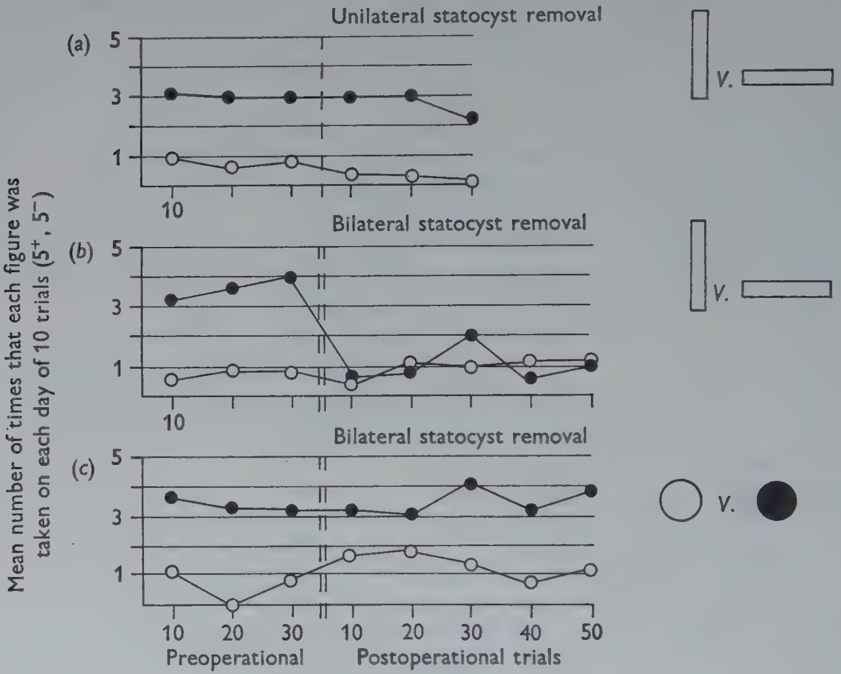

Fig. 2. The effect of statocyst removal on the performance of octopuses in visual discrimination experiments. In each plot ● shows the number of times that the positive figure was attacked and ○ the number of attacks on the negative. (*a*) A summary of the performance of eight animals from which one statocyst only was removed in the course of training; four of the animals had the left, and four the right statocyst removed; performance in the learned discrimination between horizontal and vertical rectangles was not affected by the operation. (*b*) A similar plot of the performance of ten octopuses which had both statocysts removed (four of these had the statocysts removed in two successive operations, their performance after removal of the first statocyst being included in plot *a*). Removal of both statocysts destroys the capacity to discriminate successfully between the rectangles. (*c*) A similar plot of the performance of five animals trained to discriminate between black and white disks. Removal of both statocysts does not prevent discrimination. These summaries are compounded from training experiments of variable length, and only the thirty trials immediately preceding operation and the first thirty or fifty postoperational trials are plotted. Details of some individual performances are given in Fig. 3.

vertical or nearly vertical (i.e. at right angles to the position held during preoperational training), while the other two (FK8 and FK10) generally sat with their pupils horizontal. FK7 and FK9, with pupils vertical, took the negative object more often than the positive and thus made more than random errors, while FK8

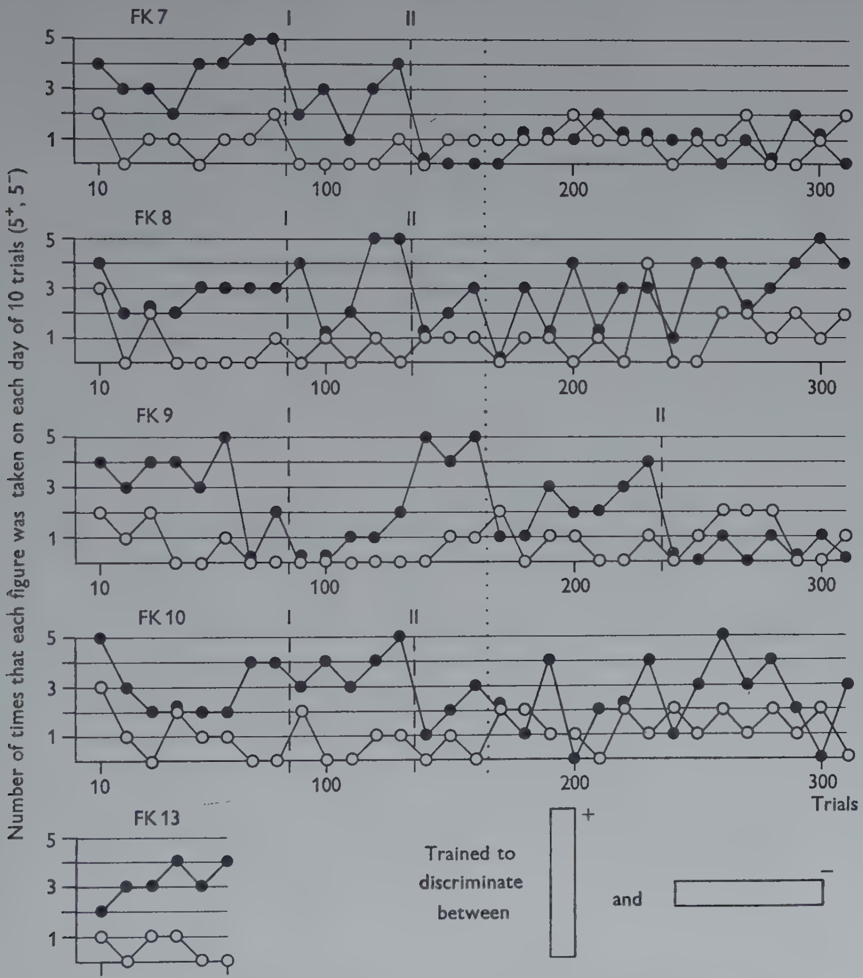

Fig. 3. Details of the performance of four octopuses from which both statocysts were removed in successive operations, and the performance of a fifth control animal. All were trained to discriminate between horizontal and vertical rectangles. The results are plotted as in fig. 2. Vertical broken lines marked I and II show when first one (LHS in the case of FK7 and FK8, RHS in FK9 and FK10) and then the second statocyst was removed. At all trials subsequent to the vertical dotted line and throughout the training of FK13 the horizontal rectangle was moved along its long axis (see text).

and FK10, with the pupils remaining predominantly horizontal, made considerably less; in preoperational training the performances of FK7 and FK9 were marginally better than those of FK8 and FK10 (Table 2). When the postoperational responses of these four octopuses are classified according to the position of the pupil at the

Table 2. *Details of postoperational attacks in training to discriminate between* ▯ *and* ▭ *(with the vertical rectangle as positive figure), considered in relation to the position of the eye used to direct the attack. Bilateral statocyst removals*

	Position of the pupil			
	—	|	/ \ or uncertain	
Correct responses/trials in which the eye was oriented as given above				
FK7	13/23	22/45	15/32	Total 49/100
FK8	37/48	13/23	21/29	61/100
FK9	11/23	16/42	8/15	35/80
FK10	42/54	9/20	13/26	64/100
Totals	103/148	59/130	57/102	—
Proportion of correct responses with the eye in the position above				
FK7	0·56	0·47	0·47	—
FK8	0·77	0·57	0·72	—
FK9	0·48	0·38	0·53	—
FK10	0·78	0·45	0·50	—
For all responses made by the four octopuses				
	0·70*	0·45	0·56	—

* Cf. 0·75 in preoperational training.

In preoperational training the proportions of correct responses made by these four animals were: 0·77, 0·74, 0·75, and 0·74 respectively.

time of the response, it is found that 70% (103) of the 148 responses made when the pupil was horizontal were correct; this is comparable with the performance of the animals in preoperational training, where 74–77% of the responses made were correct. In contrast to this, the responses made on occasions when the pupils were vertical were mainly incorrect, the animal tending to ignore the positive and attack the negative object; only 45% (59 out of 130) of the responses made on these occasions were correct (Table 2). It should be noticed that more than random errors were recorded, despite the fact that this analysis covers only the *last* 10 days of the training experiments shown in Fig. 3, and omits the first eighty postoperational trials in three out of the four experiments. During this period one of the initially perverse animals, FK7, was already relearning to make correct responses with the retina in the new, vertical, position; it was no longer attacking only the negative object (as at first, see Fig. 3) by the time the record of pupil position was begun.

Sutherland & Muntz (1959) have shown that direction of movement of rectangles is potentially a cue for discrimination of these figures in training experiments since untrained animals are more likely to attack a rectangle moving along its long axis than a rectangle moved at right angles to this when both are presented simultaneously. To find out whether this cue formed a basis for discrimination occurring after statocyst removal, the horizontal figure was moved along its long axis whenever presented in the course of the last 150 trials of the four experiments with FK animals described above, including throughout the whole of the period in which a record of pupil position was kept; performance did not appear to be altered in any way by this change in method of presentation (Fig. 3). A further unoperated animal (FK 13) was trained to discriminate between the rectangles at the same time; both rectangles were moved parallel to their long axes throughout this experiment, in which the animal learned as quickly as those trained in the more usual way, with both figures moved up and down (Fig. 3). The direction of movement seems to be irrelevant in these experiments, and cannot account for discrimination after statocyst removal.

DISCUSSION

After bilateral statocyst removal the eyes of *Octopus* no longer remain oriented with respect to gravity. The orientation of the retina, which normally remains constant with respect to gravity over a wide range of bodily positions (Fig. 1) becomes irregular, and the animal can no longer learn to discriminate between figures differing only in orientation.* Octopuses operated in this way can still, however, make black-white discriminations, and continue to distinguish between test figures and food, which is taken regularly even when the figures are not.

An analysis of postoperational responses by pretrained animals shows that the position of the retina relative to objects seen is a determining factor in the recognition of orientation; postoperationally attacks were made on the negative object (and not on the positive) when the eye used was at right angles to the position maintained during preoperational training (Table 2). This implies that postural information from pressure and stretch receptors and from the statocysts is not integrated centrally with the retinal input in the determination of orientation of figures seen. A simpler mechanism exists whereby the position of the retina relative to the external environment is kept constant as a result of the input from the statocysts; it remains constant even in blind animals (see Boycott (1960) and Dijkgraaf (1960)). Evidently the two eyes are 'locked' in some way, since removal of a single statocyst does not interfere with the orientation of either eye.

These results are interesting because of their relation to two hitherto separate lines of research upon the nervous organization of *Octopus*. They are interesting, first, because of their relation to the visual stimulus analysing mechanism. Sutherland (1957) has demonstrated that the performance of octopuses in visual discrimination experiments is consistent with the hypothesis that figures seen are classified centrally mainly on a basis of the ratio of their horizontal and vertical extents; octopuses can, for example, be taught to discriminate between horizontal and vertical rectangles, but not between the same rectangles shown at right angles, but obliquely. Sutherland (1957) further found that oblique rectangles are more readily distinguished from vertical than from horizontal rectangles, a finding that is explicable if the analysing mechanism at work sums more accurately in one direction than in the other *and* the orientation of the receptors is fixed relative to horizontal-vertical in the external world. (If information about the orientation of the retina were fed into the analysing mechanism *after* computation of the apparent vertical and horizontal ratios there is no reason why ╲ *v*— should be any more difficult than ╲ *v* |).

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* The slight improvement in postoperational performance by such initially perverse animals as FK 7 and FK 9 (Fig. 3) indicates, however, that learning to discriminate is potentially possible provided that the animal is always in the same position at the beginning of training trials and provided that figures are presented sufficiently close for the animal to take them without moving the head—i.e. under conditions in which the absence of the statocysts does not upset the constancy or orientation of the eyes relative to the figures.

Young (1960*b*) has recently shown an anatomical basis for the visual classification mechanism: the retinal rods are arranged in vertical and horizontal rows and are backed by predominantly vertical dendritic connexions within the optic lobes. It will be noticed that this arrangement is not only along the predicted axes, but is also asymmetric in the expected direction.

The eye-statocyst relationship is also interesting because it confirms what we already suspect about the role of proprioception in learning by cephalopods. Wells & Wells (1957) showed that octopuses can readily be trained to discriminate between objects differing in degree of surface irregularity (roughness) but not between objects differing only in the pattern or orientation of surface irregularities —they cannot be trained to make discriminations that depend upon taking into account the relative position of receptors on the arms. Moreover, attempts to teach octopuses to discriminate between objects differing in weight (Wells, 1961) have so far failed, which again implies that proprioceptive information (this time potentially available from 'stretch' receptors in the arms) is not available as a basis of learned adjustments in behaviour. The observation of Boycott (1960) that octopuses do not learn to use other proprioceptive information about the position of the arms and body in order to orientate themselves after statocyst removal has been repeated several times in the course of the present series of experiments, and would appear to confirm the over-all impression that proprioceptive information is not available for learning, although it clearly must contribute to many essentially reflex activities in movement and in the maintenance of posture (see, for example, Boycott & Young, 1950). On the evidence now available proprioceptive information from the statocysts would appear to be no exception to this general rule. This information is clearly used in reflex adjustments of the position of the eyes (see, for example, Dijkgraaf (1960) on the effects of rotation upon eye movements in blinded animals), but there is no need to suppose that it is available for central integration with other sensory inputs.

SUMMARY

1. Twenty-two small octopuses were trained to make visual discriminations before and after removal of the statocysts.

2. Removal of one statocyst (left or right) did not affect performance in a visual discrimination of rectangles shown horizontally and vertically (8 experiments), nor did it determine which eye was used to guide attacks upon things seen (a record of this was kept in four of the experiments).

3. Removal of both statocysts did not affect performance in a discrimination between black and white circles (7 animals) but did destroy the capacity to discriminate successfully between rectangles shown horizontally and vertically (11 animals).

4. After removal of both statocysts the orientation of the retina (as indicated by the slit-like pupils) is no longer constant over the normal wide range of bodily positions, but depends upon how the animal is sitting on the side or bottom of its tank.

5. An analysis of the postoperational responses of animals trained to make the orientation discrimination showed that nearly all of them were biased in favour of one or other of the test figures, not always that used as 'positive' figure during preoperational training. The postoperational scores of these trained animals depended mainly upon the positions in which they habitually sat in their tanks; individuals in positions such that the retina of the eye used to observe the test figures lay at right angles to the position held during pretraining tended to behave perversely.

6. These results indicate that orientation discrimination is dependent upon correct orientation of the retina, rather than upon central integration of proprioceptive and visual information. This is discussed in relation to a possible mechanism of visual discrimination proposed by Sutherland (1957), and in relation to what is already known about the role of proprioceptive information in learning.

The author would like to thank the director and staff of the Stazione Zoologica di Napoli for their hospitality while these experiments were being made. He would also like to thank Mr J. M. B. Messenger, who helped by training most of the EK (1958) series of animals, and Prof. J. Z. Young, F.R.S., for his comments upon the work in manuscript.

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THE NUTRITION OF THE CENTRAL NERVOUS SYSTEM IN THE COCKROACH *PERIPLANETA AMERICANA* L.

THE ROLE OF PERINEURIUM AND GLIAL CELLS IN THE MOBILIZATION OF RESERVES

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(With Plate 13)

The organs and tissues of insects are mostly composed of single layers of cells separated from the circulating blood by thin connective tissue membranes (Wigglesworth, 1956*a*; Pipa & Cook, 1958). The supply of nutrients to such organs presents no problem. But the central nervous system is an exception. The ganglia are the only solid organs in the insect body. They consist of densely packed peripheral cells surrounding a central neuropile, the whole ganglion being covered by a tough fibrous sheath or neural lamella continuous with the connective tissue membranes elsewhere. The total thickness of the ganglion may be 1 mm. or more.

The need for oxygen is met by the tracheae and tracheoles which pass through the neural lamella and ramify inside. But there is no circulation of haemolymph within the ganglion. All nutrient substances must diffuse through the neural lamella and be passed on to the deeper layers. One would, therefore, expect to find some special provision for the transport and storage of such nutrients.

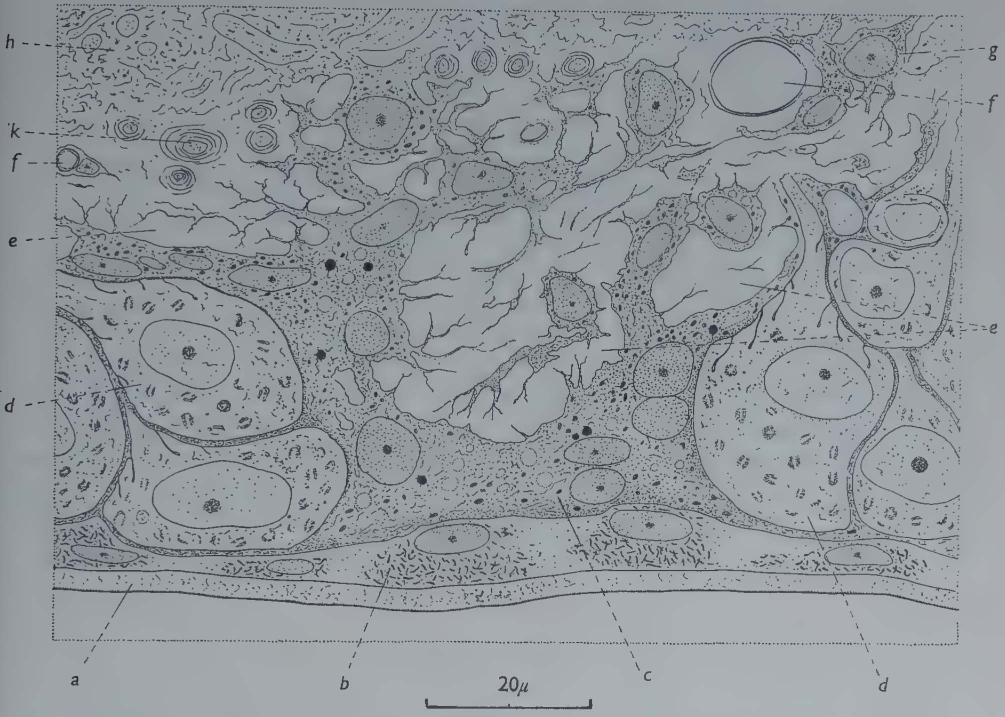
After passing through the neural lamella the nutrient substances must be taken up by the perineurium cells, transferred to the glial cells, and conveyed by these to the neurones. In an earlier paper (Wigglesworth, 1959*b*), the probable importance of both perineurium and glial cells in the nutrition of the neurones was emphasized.

The present paper, which deals with histology and histochemistry, is part of a general study of the nutrition of the nervous system in the cockroach. The biochemical observations are published separately by Treherne (1960).

METHODS

Most of the work has been done on the last abdominal ganglion of the adult male cockroach, *Periplaneta americana*, with the cercal nerves behind and the connectives in front. The aim has been to relate the anatomy and histology of the ganglion to the deposition of visible reserves of triglycerides and glycogen under different conditions of nutrition.

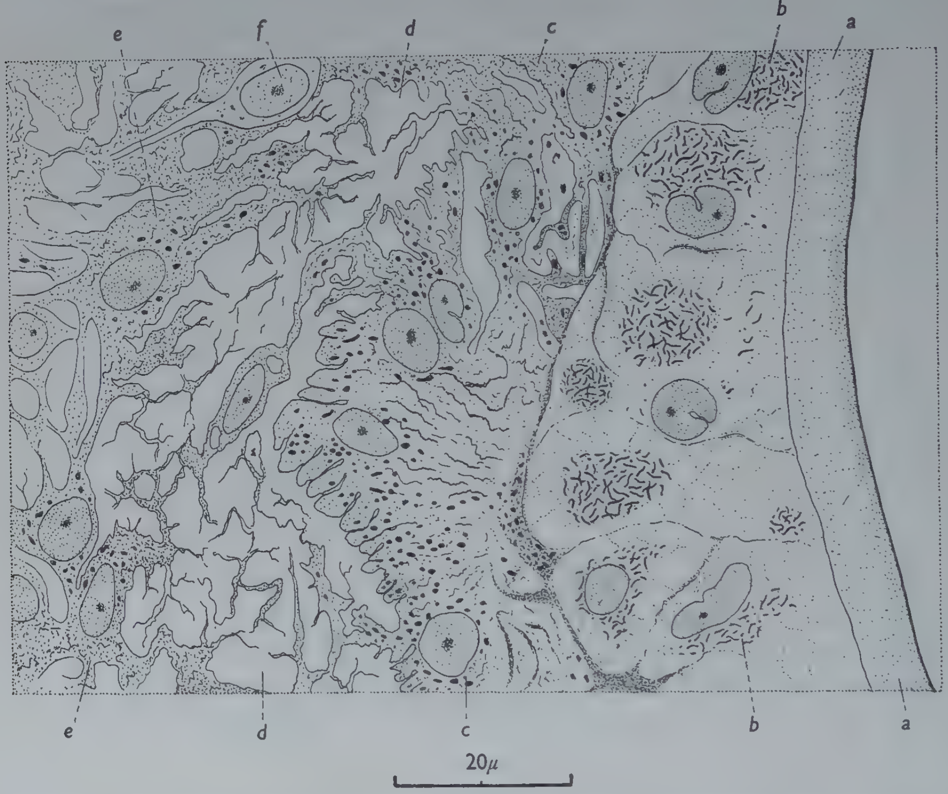
For general histology the osmium tetroxide and ethyl gallate method of fixation and staining has been used, horizontal and transverse sections at 0·42–2 μ being cut by the method already described (Wigglesworth, 1959*c*). This same method reveals triglycerides as blue-black droplets. For glycogen the periodic acid-Schiff test (with saliva-treated controls) has been used after fixation with alcoholic Bouin, Hansen's iron haematoxylin and light green being employed as counterstains.



Text-fig. 1. Horizontal section (2 μ) through the lateral margin of the terminal ganglion (osmium tetroxide and ethyl gallate). *a*, neural lamella; *b*, perineurium cells with massed mitochondria; *c*, outer glial cells with mitochondria, small fat droplets and vacuoles in the cytoplasm; *d*, large ganglion cell with Golgi bodies; *e*, glial lacunar system with glial cell filaments: *f*, trachea; *g*, inner glial cells cells applied to the neuropile; *h*, neuropile; *k*, axons with concentric sheaths.

ANATOMY AND HISTOLOGY OF THE GANGLION: THE GLIAL LACUNAR SYSTEM

The osmium and ethyl gallate method reveals many new features in the ganglion, but the present description will be limited to those points that are relevant to the problem in hand. The thick laminated neural lamella containing collagen has been described by Baccetti (1955, 1956) and by Hess (1958*a*, *b*). The perineurium cells, as in *Rhodnius* (Wigglesworth, 1959*b*), contain densely packed masses of filamentous mitochondria (Hess, 1958*b*) (Text-figs. 1, 2). These cells are described by Hess (1958*b*) as cubical. Actually they vary in height in different regions of the ganglion. Along the sides they are somewhat flattened, say 3–6 μ (Text-figs. 1, 6D), but at the anterior and posterior extremities of the ganglion, particularly on the dorsal aspect, they form a columnar epithelium that may be 20–30μ in height (Text-figs. 2, 6B, C); and all intermediate conditions occur.



Text-fig. 2. Horizontal section (2 μ) through the anterior end of the ganglion just dorsal to the connectives (osmium tetroxide and ethyl gallate). *a*, neural lamella; *b*, perineurium cells with clumps of filamentous mitochondria; *c*, outer glial cells with oval and rod-like mitochondria; *d*, glial sinus system, partially collapsed; *e*, inner glial cells bounding the neuropile; *f*, small neurone.

Beneath the perineurium is the glial cell layer (Text-figs. 1, 2). This consists of two ill-defined parts; a peripheral part which invests the ganglion cells where these are present, and a central part which surrounds the neuropile. The glial cells contain oval and short rod-like mitochondria. These are much stouter than the filamentous mitochondria of the perineurium cells. Giant glial cells, the type iii as described in *Rhodnius*, do not occur in *Periplaneta*. Tracheae and tracheoles are most abundant in the central part of the glial zone, adjacent to the neuropile (Text-fig. 1).

The glial cell layer is more or less vacuolated. The vacuoles appear to arise within the cytoplasm. But in many ganglia the vacuolation is so extreme that a wide space or sinus separates the two layers of glial cells. This 'glial lacunar system' is commonly most evident on the dorsal side of the ganglion, but it also extends laterally and may surround the neuropile on all sides (Pl. 13, figs. 1–3). In its fully developed state the glial nuclei surrounded by scanty cytoplasm lie within extensive cavities which are traversed in all directions by slender branching filaments or trabeculae (Text-fig. 1). Along with the glial nuclei are the much smaller nuclei of tracheal cells supporting tracheae and tracheoles which are suspended in the sinus cavities by delicate mesenteries.

The contents of these vacuoles or lacunae appear virtually colourless after staining with osmium and ethyl gallate. But the air-containing tracheae show up in the sections as a brighter white than the lacunar spaces, which evidently contain a very tenuous plasma. A vacuolated space of the same type is present in the small abdominal ganglia and in the thoracic ganglia.

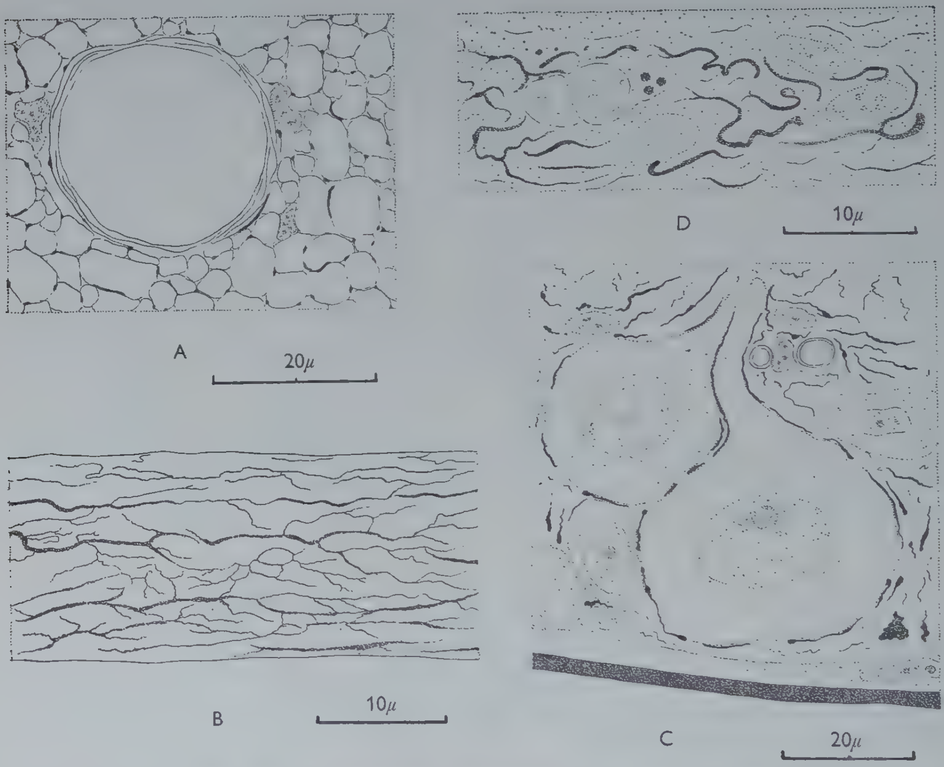
The function of the glial lacunar system is uncertain. It might serve as a pool into which nutrients are discharged and from which they can be drawn by those cells which require them. Its development is very variable. In some insects the cavities are collapsed: they are little more than potential spaces among the glial cells (Pl. 13, fig. 4). This condition seems to be characteristic of young and well-nourished insects. In old or in starved insects the spaces are generally large (Pl. 13, fig. 3). This suggests that perhaps the lacunar system is simply a provision for maintaining unchanged the outward form of the ganglion, as defined by the thick fibrous neural lamella, while the cellular structures within the ganglion expand or shrink according to their state of nutrition. This curious structure must often have been observed before; it can be seen, for example, in the terminal ganglion of *Periplaneta* figured in longitudinal section by Roeder (1948); but I have found no description of it.

THE GLIAL CYTOPLASM; PAS-POSITIVE STRUCTURES

The glial cytoplasm extends everywhere between the ganglion cell bodies, and between the nerve fibres in the neuropile and in the nerves and connectives (as already described in *Rhodnius*). If the nervous system is fixed with alcoholic Bouin, and glycogen is removed from the sections with saliva before treatment with PAS, the neural lamella and the glial cytoplasm give a positive reaction (Wigglesworth, 1956*a*).

In the glial cytoplasm the PAS-positive material takes several forms. (i) Irregular deposits, presumably reserve material to be used in the formation of glial membranes, etc. (Text-fig. 3 C). (ii) Delicate homogeneous membranes around the cell bodies and axons, and lining the glial cavities (Text-fig. 3 A, C). Around the giant axons in the connectives these sheaths are multiple (Text-fig. 3 A). (iii) Tapering and branching strands in the cytoplasm of the glial cells (Text-fig. 3 C, D). These may occupy the larger filaments which traverse the spaces in the glial lacunae. They occur as thickenings in the membranes around the ganglion cells and axons. Here they appear in cross-section as purple points around the axon sheaths (Text-fig. 3 A). In surface view of the large axons they appear as a network of fibrils (Text-fig. 3 B). This arrangement was described by Pipa & Cook (1958) in the nerves of the sucking louse.

All these structures are presumably composed of the collagen-like material made up of banded fibrils that has been described in the neural lamella (Hess, 1958*a*; Smith & Wigglesworth, 1958) and in the glial cytoplasm within the ganglia (Gray, 1959). Besides giving a positive PAS reaction they stain with haematoxylin (slightly) and with light green. As a result they have a purple colour in the sections which, even in the absence of the saliva test, serves to distinguish them from glycogen, which is stained crimson.

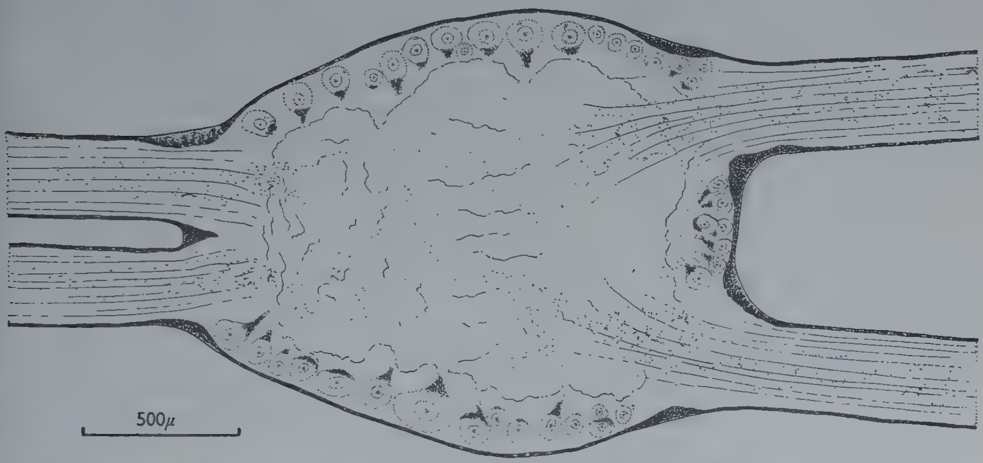


Text-fig. 3. Sections through the ganglion and connectives (4 μ) (alcoholic Bouin; PAS after saliva) showing PAS-positive structures. A, Transverse section of connective; B, horizontal section of connective showing large axon sheath in surface view; C, lateral margin of ganglion showing neural lamella and PAS-positive deposits, membranes and fibrils in the glial cytoplasm; D, detail of glial cells adjacent to the neuropile, showing branching fibrils in the cytoplasm.

DISTRIBUTION OF GLYCOGEN IN THE GANGLION AND NERVES

Text-fig. 4 shows the general distribution of glycogen in the ganglion of the well-nourished insect. It is most conspicuous in the perineurium cells which are often stuffed with glycogen like fat-body cells. It is scanty in the glial cytoplasm, and it occurs as a fine 'dust', barely resolvable under the oil immerion, in the glial membranes which ensheath the axons in the connectives and cercal nerves, and in the glial component of the neuropile.

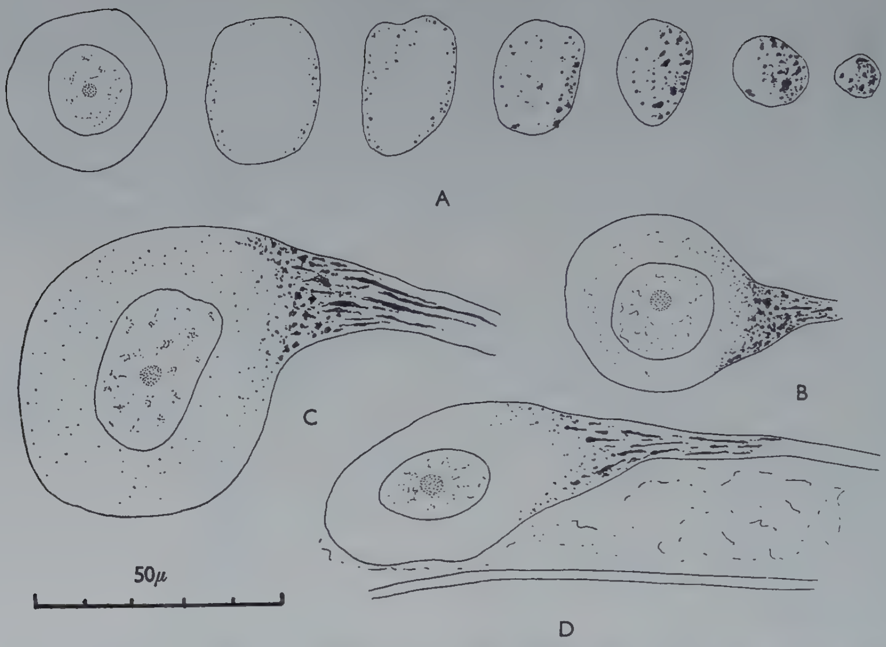
The glycogen in the glial cytoplasm is most evident at the base of the cercal nerve and this gives the impression that it is being transmitted from the ganglion to the nerve. In the cercal nerves and in the connectives the cells below the neural lamella combine the functions of perineurium cells and Schwann cells (cf. Hess, 1958*a*; Wigglesworth, 1959*a*). They contain glycogen, as in the specialized perineurium cells of the ganglia, and they presumably transmit this glycogen into the deeper parts of the subjacent nerve, where traces are detectable as a fine 'dust' in the axon sheaths.



Text-fig. 4. Semi-schematic scale drawing of terminal ganglion in horizontal section with connectives to the left and cercal nerves to the right, showing distribution of glycogen in perineurium cells of ganglion and nerves, in the inner aspect of the large ganglion cells and in the glial component of neuropile and nerves.

Glycogen is conspicuous in the nerve cells; it lies predominantly on the inner side where the axon is given off. It is well known that in tissues fixed with alcoholic fixatives, glycogen, even more than other cell components, may be swept forwards by the advancing fixative and become concentrated on the opposite side of the cell. The question arises whether the accumulation of glycogen in the axon cone is an artifact of fixation or represents the distribution in the living cell.

There seems little doubt that it is not merely an effect of fixation. (i) The distribution is unchanged if the ganglion is split open so that the fixative can enter the cells from both sides. (ii) In normal ganglia fixed with alcoholic Bouin glycogen is likewise concentrated in the axon cone in those cells in which the axon lies parallel with the surface of the ganglion (Text-fig. 5 A, D). But it will be noted in Text-fig. 5 A that there has been some displacement of glycogen during fixation. (iii) If the ganglion is exposed for 10 min. in buffered osmium tetroxide before transfer to alcoholic Bouin, the cytological fixation is good with no evidence of displacement, but the distribution of glycogen in the ganglion cells is the same; it is concentrated in the axon cone.



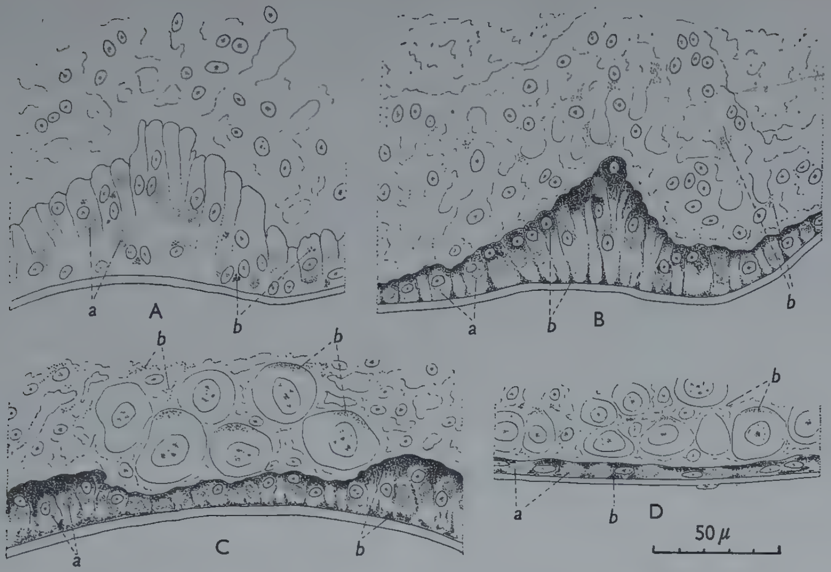
Text-fig. 5. Ganglion cells from cockroach 24 hr. after injection of 0·2 ml. of 6 % glucose, showing glycogen. A, Successive 4 μ sections of a single cell showing progressive increase in glycogen towards the axon. The surface of the ganglion lies to the left of the figures; note the displacement of glycogen towards the right. B, C and D, longitudinal sections; in D the origin of the axon lies parallel with the neural lamella, shown below.

GLIAL INVAGINATIONS AND THE TRANSFER OF CARBOHYDRATE TO THE GANGLION CELLS

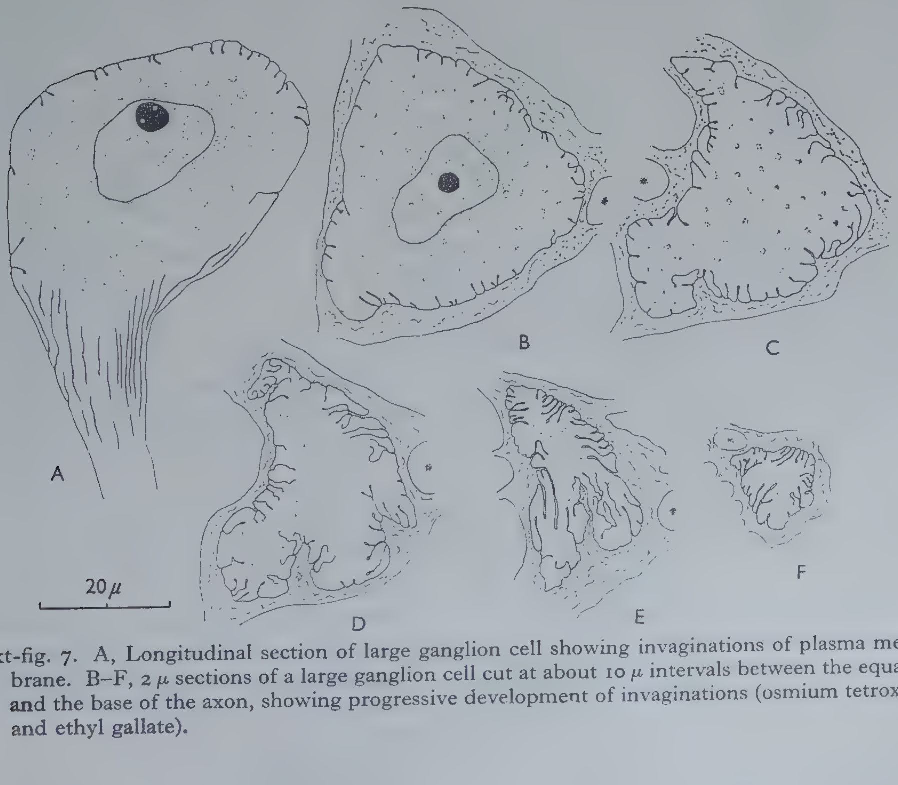
The cell membrane of the large ganglion cells in insects is invaginated so that the glial cytoplasm penetrates deeply into the body of the cell. This arrangement was termed by Holmgren (1900) the 'Trophospongium'. In *Rhodnius* also it was interpreted as the mechanism by which the glial cells furnish nutrient substances to the ganglion cells (Wigglesworth, 1959*b*). It was of interest to see whether there is any visible relation between the glial invaginations and the transfer of glycogen.

Text-fig. 7 A shows a longitudinal section through a large ganglion cell fixed and stained with osmium and ethyl gallate. There are small invaginations from all parts of the cell surface, but these are much more conspicuous and numerous in the axon cone. Text-fig. 7 B–F represents a series of transverse 2 μ sections cut at intervals of 10–12 μ from the equator of a large ganglion cell to the beginning of the axon. It shows the progressive development of glial invaginations as the axon cone narrows to form the axon. These drawings also indicate the thickness of the layer of glial cytoplasm that encloses the ganglion cell at the different levels.

Thus the accumulation of glycogen in the ganglion cell goes hand in hand with the development of the glial invaginations. Moreover, longitudinal sections through the beginning of the axon (Text-fig. 5 C, D) commonly show the glycogen



Text-fig. 6. A, Horizontal section of the anterior end of the terminal ganglion just above the connectives, from a cockroach starved for 3 weeks (alcoholic Bouin, PAS to show glycogen). Perineurium cells contain clumps of mitochondria and traces of glycogen. B, The same in starved cockroach 3 hours after feeding on honey and serum. Much glycogen in perineurium cells and traces in glial cells. C, same insect as B, posterior end of the ganglion showing glycogen in ganglion cells and in glial cytoplasm. D, The same, lateral margin of the ganglion. *a*, Clumps of mitochondria; *b*, glycogen.



Text-fig. 7. A, Longitudinal section of large ganglion cell showing invaginations of plasma membrane. B–F, 2 μ sections of a large ganglion cell cut at about 10 μ intervals between the equator and the base of the axon, showing progressive development of invaginations (osmium tetroxide and ethyl gallate).

deposits in elongated strands, strongly suggesting that it is contained within or on the surface of the invaginations. This impression is confirmed by the examination of sections of ganglia fixed for 10 min. in osmium tetroxide before transfer to alcoholic Bouin. These show the glycogen between the glial membranes in some cases, applied to the surface in others.

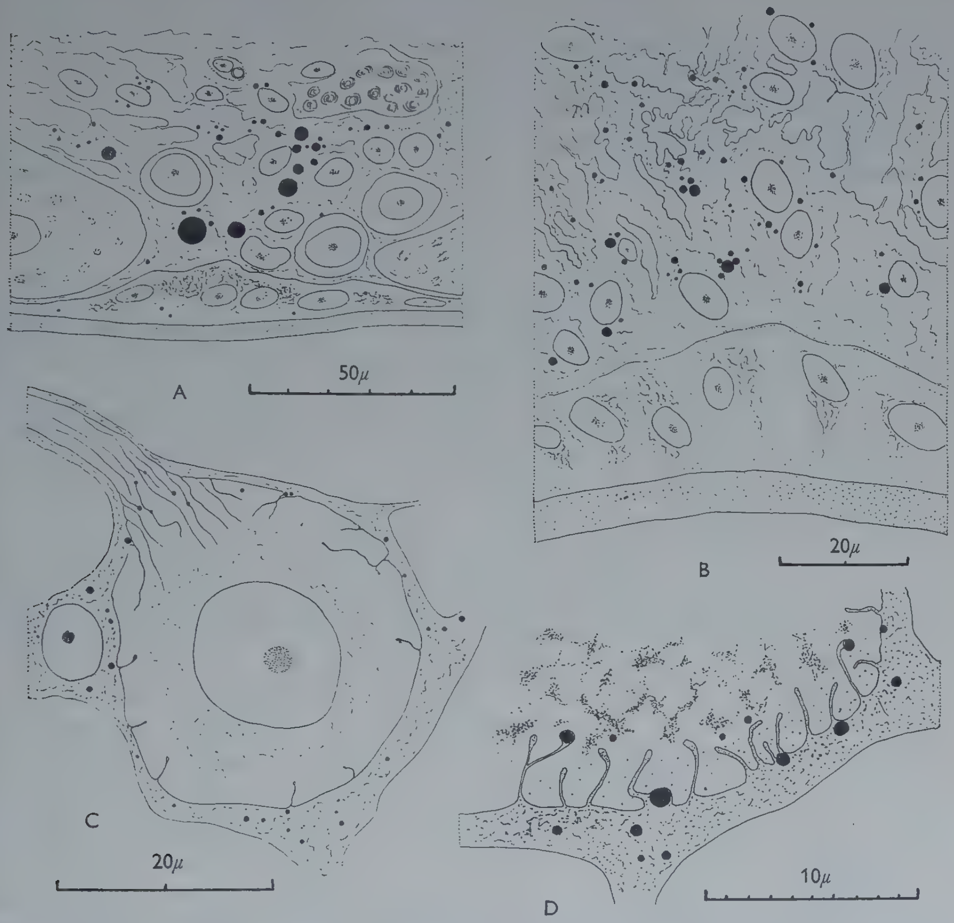
It might be argued that since the ganglion cells are known to be continuously secreting the axon contents, the deposition of glycogen towards the base of the axon is merely a reflexion of this movement. But glycogen appears in this situation very rapidly after feeding. Young adult male cockroaches have been kept on water alone with no food for 3–4 weeks at 26° C. At the end of this time the fat body is devoid of glycogen and contains only small droplets of fat. Glycogen has practically disappeared from the ganglion; only occasional minute traces are detectable in the perineurium cells (Text-fig. 6 A). But within 3 or 6 hr. after the cockroach has been given a meal of honey the glycogen content of the ganglion has been restored and the deposits in the ganglion cells are again concentrated in the axon cone with no more than a fine 'dust' in the remainder of the cytoplasm. In these preparations fixed with alcoholic Bouin and stained with PAS and Light Green, the mitochondria in the perineurium cells appear as green-staining masses. The glycogen is confined to the periphery of the cell and is entirely absent from the clumps of mitochondria (Text-fig. 6 B–D).

DISTRIBUTION OF TRIGLYCERIDE IN THE GANGLION

The ganglia and nerves contain large amounts of lipid in the axon sheaths, in the numerous mitochondria, and in the Golgi bodies; it is this lipid which is mainly responsible for the uptake of osmium and so for the deep coloration with ethyl gallate (Wigglesworth, 1957). The greater part of these lipids are presumably phospholipids which give a grey-brown coloration with osmium and ethyl gallate. Triglycerides appear as blue-black spheres. They are present in much smaller quantity, but they do occur in the cytoplasm of the glial cells in the well-nourished insect (Text-fig. 1). They range from droplets about 2 μ in diameter to fine 'dust-like' particles, and occasional minute droplets of fat appear in the cytoplasm of the ganglion cells and perineurium.

As in *Rhodnius* (Wigglesworth, 1959*b*) the glial invaginations in the ganglion cells often contain deeply staining lipid material in their inner extremities; and darkly staining inclusions are most evident in the immediate neighbourhood of the invaginations. This localized accumulation of minute lipid droplets over the surface of the glial invaginations persists even in insects starved for 3–4 weeks. But the relation of fat droplets to the invaginations is most evident when the deposition of fat in the ganglion is exaggerated by the injection of large amounts of sugar.

Text-fig. 8 shows sections of a ganglion 20 hr. after the injection of 0·2 ml. of 10% glucose into a normally nourished male cockroach. At the anterior end of the ganglion, just dorsal to the connectives, the high columnar perineurium cells contain no visible deposits of fat, but the underlying glial cells contain abundant small droplets (Text-fig. 8 B). At the margin of the ganglion (Text-fig. 8 A) a few minute droplets of fat are present in the perineurium, but much greater quantities are in the cytoplasm of the glial cells.



Text-fig. 8. Sections of ganglion of cockroach 20 hr. after injection of 0·2 ml. of 10 % glucose (osmium tetroxide and ethyl gallate). A, Side of ganglion showing a few minute fat droplets in the perineurium cells, numerous droplets in glial cytoplasm (2 μ section). B, Horizontal section of anterior end of ganglion; fat droplets absent from perineurium cells, plentiful in glial cytoplasm. (Mitochondria faintly indicated in the perineurium but omitted from the glial cells.) C, Ganglion cell surrounded by glial cytoplasm. Minute fat droplets in glia and within the plasma membrane invaginations towards the axon. D, Detail of invaginations and fat droplets in part of a ganglion cell (0·85 μ section).

Text-fig. 8 C shows a longitudinal section of a large ganglion cell. As usual, the invaginations from the glial cells are conspicuous in the axon cone and minute blue-black droplets of fat are associated with them and appear to lie between the invaginated cell membranes.

Text-fig. 8 D shows the details of invaginations on the inner aspect of a large ganglion cell. Droplets of fat are plentiful in the glial cytoplasm; they occur in the base of some of the invaginations into the ganglion cell; and they often occur between the membranes deep in the body of the cell. An occasional droplet may appear to lie free in the cytoplasm; but it is difficult to be sure whether these are droplets which have been liberated from the glial invaginations or whether they are still enclosed within invaginations whose connexion with the cell surface is not visible in that particular section.

The total quantity of visible fat droplets deposited in the ganglion cell is very small in comparison with the glycogen. But the glial invaginations are again clearly concerned in their transfer.

PERMEABILITY OF THE PERILEMMA

The perilemma is the term used for the total sheath of the ganglion, embracing the inner cellular layer, the perineurium, and the outer fibrous sheath, the neural lamella. It is usually suggested that the neural lamella is responsible for the selective impermeability of the total sheath. But the evidence set out in the earlier paper on the ganglia of *Rhodnius* (Wigglesworth, 1959*a*) favoured the view that the neural lamella is freely permeable and that it is the cellular perineurium which regulates the passage of solutes into the ganglion.

Observations made during the present work support that conclusion. Trypan blue (0·2 ml. of 0·5% solution in Ringer) was injected. The ganglion was later fixed with Carnoy and the sections stained with Orange G. At 2 days after the injection the neural lamella was coloured a diffuse blue. More dye was concentrated in haemocytes spread out on the surface of the ganglion. At 7 days after the injection there were very small amounts of dye in the perineurium cells and still smaller amounts in the glial cells. There seemed to be no absolute distinction in this respect between the perineurium cells and glial cells (such as was described by Scharrer, 1939); the difference is in degree only. The pericardial cells and dermal glands take up most of the dye; the haemocytes and fat body come next; the perineurium cells take up much less and only traces reach the glial cells.

It would appear that the neural lamella has a sponge-like structure which readily admits even rather large molecules like trypan blue. These results are comparable with those of Röhlich & Weiss (1955) which confirmed in the sciatic nerve of the rat the earlier opinions of Retzius and of Ranvier that the resistance to entry of iron salts is dependent on the cellular layer of the perineurium, the connective tissue sheath being freely permeable (cf. Hoyle, 1953).

DISCUSSION

The glial cells in the ganglia of insects have long been regarded as 'trophocytes' with the function of nourishing the neurones. The results here described demonstrate the role of the perineurium cells (which are regarded as a specialized type of glial cell (Wigglesworth, 1959*b*)) in the storage of glycogen, and the role of the glial cells proper in transferring glycogen and fat (or the precursors of these reserves) to the ganglion cells by way of the plasma membrane invaginations or 'trophospongium'. These reserves, particularly the glycogen, are concentrated in the ganglion cell towards the base of the axon. That suggests that they contribute to the secretion from the body of the ganglion cell which is believed to pass continuously into the axon.

A large part of the nutrients entering the ganglion cell will be small molecules, glucose or trehalose and the intermediary metabolites derived from these (Treherne, 1960). Such substances cannot be located by the procedures described in this paper; but the movements of glycogen and fat which follow massive ingestion or injection of sugars may perhaps serve to indicate the route they must follow in reaching the neurones.

The perineurium cells and the glial cytoplasm of *Rhodnius* are very rich in enzymes: 'esterase' of various types (Wigglesworth, 1958), succinic dehydrogenase (Wigglesworth, 1956*b*); and since these properties are common to the perineurium cells of the ganglion and of the peripheral nerves, a nutritional function was assumed to be common to both (Wigglesworth, 1959, *a*, *b*). F. O. Schmitt (1958) emphasized the high metabolic and enzymic activity of the satellite cells (glial cells, Schwann cells) in the peripheral nerves of mammals. He attributes to these cells a 'nutritional function', to provide for local metabolic energy and perhaps also the type of energy necessary for ion pumping.

The present work demonstrates the role of the glial cells in the massive transfer of carbohydrates and fats to the neurones in the central nervous system. There is some visible transfer also in the large nerves and connectives. It seems probable that this nutritive function is general throughout the nervous system.

SUMMARY

1. The histology of the last abdominal ganglion and the cercal nerves and connectives of the cockroach are briefly described. Attention is called to the large cavities, termed the 'glial lacunar system', that are present in the glial cell layer of the ganglion; and to the branching filaments of collagen-like material which are laid down within the glial membranes and trabeculae of the ganglia and nerves.

2. Glycogen is stored in large amounts in the perineurium cells, and in small amounts in the interaxonal glial membranes in the neuropile and nerves. Invaginations of the plasma membrane of the large ganglion cells (the 'trophospongium') are apparently concerned in the transfer of glycogen. Invaginations and glycogen deposits increase progressively towards the base of the axon.

3. Very small amounts of triglycerides are stored in the ganglion. There are traces only in the perineurium cells; rather more in the glial cells. The invaginations of the glial cells into the large ganglion cells seem to be concerned also in the transfer of lipids to the neurones.

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EXPLANATION OF PLATE

All sections fixed and stained with osmium tetroxide and ethyl gallate, and cut at 2 μ.

Fig. 1. Horizontal section of terminal ganglion slightly dorsal to the cercal nerves and connectives, showing, from without inwards, the layer of ganglion cells, the clear 'glial lacunar system', and the darkly staining neuropile.

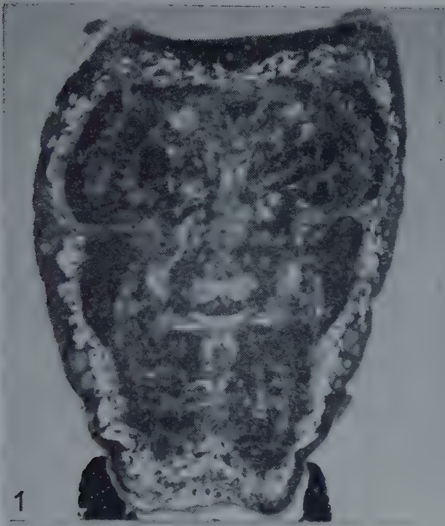
Fig. 2. The same as fig. 1, cut at a more dorsal level: glial lacunae very extensive.

Fig. 3. Transverse section of terminal ganglion from an old and starved cockroach: glial lacunar system well developed.

Fig. 4. The same as fig. 3, from a young and well-nourished cockroach: glial lacunae largely collapsed.

Fig. 5. Detail of the glial lacunae from the ganglion represented in figs. 1 and 2 above, showing glial cells connected by branching trabeculae.

Fig. 6. Large ganglion cells invested by glial cells, and with their axons crossing the glial lacunae. The outer limit of the neuropile is seen to the left below.



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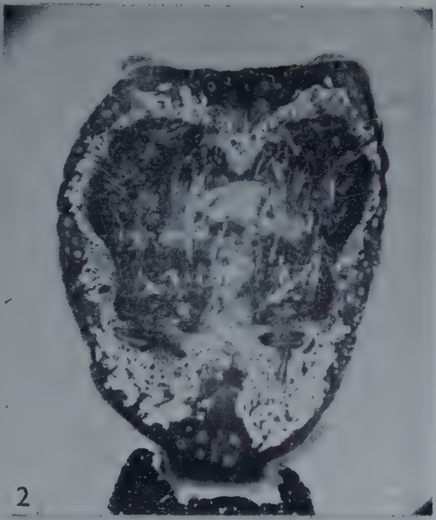

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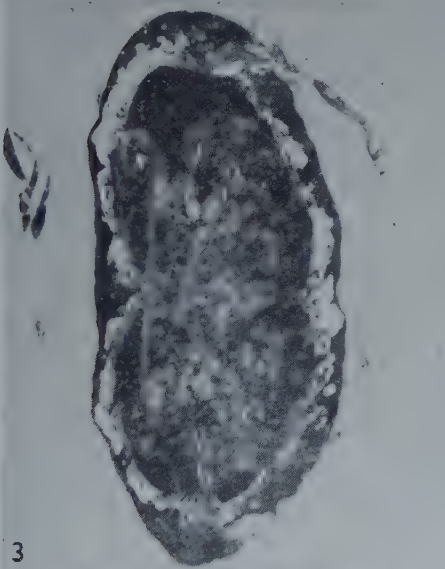

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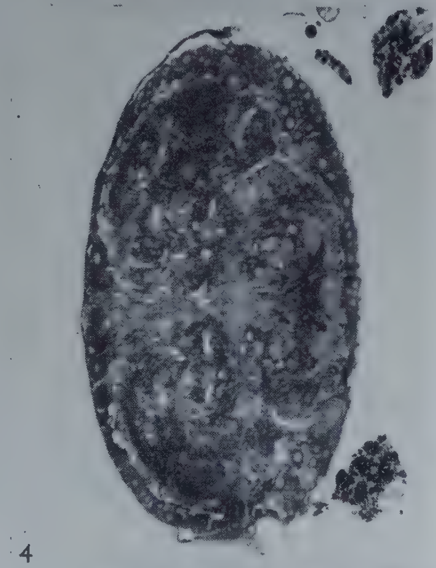

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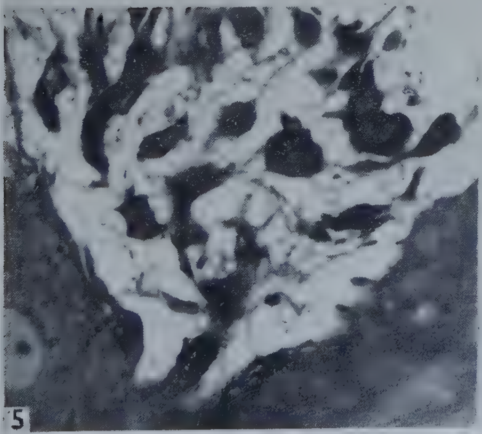

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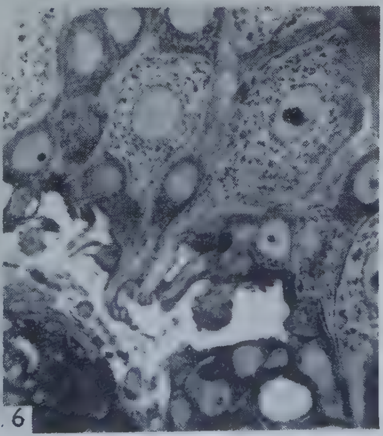

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WIGGLESWORTH—THE NUTRITION OF THE CENTRAL NERVOUS SYSTEM IN THE COCKROACH *PERIPLANETA AMERICANA* L. THE ROLE OF PERINEURIUM AND GLIAL CELLS IN THE MOBILIZATION OF RESERVES

(*Facing p.* 512)

THE NUTRITION OF THE CENTRAL NERVOUS SYSTEM IN THE COCKROACH, *PERIPLANETA AMERICANA* L.

THE EXCHANGE AND METABOLISM OF SUGARS

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INTRODUCTION

The insect central nervous system is enveloped by a continuous cellular and fibrous membrane, the perilemma, which has been shown to be a barrier to the inward diffusion of sodium and potassium ions (Hoyle, 1952, 1953; Twarog & Roeder, 1956), acetylcholine (Twarog & Roeder, 1956) and dyes (Wigglesworth, 1960). On the other hand, this structure must also, as Wigglesworth (1959, 1960) has pointed out, be specialized to allow exchanges of nutritive and excretory substances to take place between the central nervous system and the haemolymph. This important role of the perilemma has been little studied and no quantitative observations have been made on the entry of nutritive substances into the insect central nervous system. In this investigation an attempt has been made to throw some light on these processes by studying the uptake of some ^{14}C-labelled sugars in the abdominal nerve cord of the cockroach.

Relatively little is known of the biochemical events occurring within the insect central nervous system, and the opportunity has been taken to learn something of these processes by following the metabolic fate of the ^{14}C-labelled compounds in the abdominal nerve cord of this insect. These observations form part of a wider investigation being carried out in this laboratory in collaboration with Prof. V. B. Wigglesworth. The parallel histological and histochemical aspects of this problem are dealt with in a separate study on the central nervous system of this insect (Wigglesworth, 1960).

METHODS

The adult male *Periplaneta americana* L. possesses a nerve cord which is relatively free from associated fat body, and for this reason is particularly suitable for use in an investigation of this kind.

In these experiments the uptake and subsequent metabolism of ^{14}C in the nerve cord was studied after the injection of radioactive glucose into the haemolymph. The injected glucose, which was generally labelled with ^{14}C, was dissolved in Hoyle's saline (Hoyle, 1953) and had a specific activity in the range 3·77–25·7 mc./mM. In each case 10·0 μl. of the radioactive solution was introduced into the haemolymph, with an Agla syringe, through an abdominal intersegmental membrane, the punctured cuticular surface being afterwards sealed with wax. After a given experimental period the insect was anaesthetized with CO_2, some haemolymph collected for analysis and the whole abdominal nerve cord quickly removed by dissecting through the ventral abdominal surface. Care was taken to ensure that all adhering tissues were removed from the nerve cord and those which showed excess amounts of fat body in the region of the ganglia were rejected. The cut ends of the isolated nerve cord were gripped with fine watch-maker's forceps and the whole preparation was washed in ice-cold saline for an appropriate period, the duration depending on the nature of the experiment. On removal from the saline the nerve cords were lightly blotted, plunged into liquid nitrogen and then transferred to a glass homogenizer immersed in liquid nitrogen, for extraction with three changes of N formic acid in 50% aqueous ethanol (Heslop & Ray, 1958). Further extraction of the nerve cords with boiling 30% KOH for 6 hr. yielded only negligibly small amounts of radioactivity.

The uptake of ^{14}C-labelled compounds was also studied in some isolated preparations. In these experiments whole abdominal nerve cords were quickly removed from the insects and placed in oxygenated saline containing trehalose and glucose in concentrations similar to those in the haemolymph. A connective, usually that between the fourth and fifth abdominal ganglia, was then gripped at both ends with automatically operated watch-maker's forceps (Heslop & Ray, 1959) in which the lateral movements were obtained by sliding ground-glass plates lubricated with petroleum jelly (Goldacre, 1954) (Fig. 1). The isolated connective was then transferred to a radioactive solution by submerging the tips of the forceps beneath the surface of the liquid. The solution was stirred with a fine jet of water-saturated oxygen or compressed air. In other experiments the last abdominal ganglia and whole nerve cords were isolated in this way. With some isolated preparations a radioactive haemolymph solution was prepared by injecting ^{14}C-labelled glucose into a cockroach and, after 40 min., removing the haemolymph from the base of the prothoracic leg with a silicone-lined pipette. The haemolymph was then precipitated with 2 vol. of absolute ethanol (to remove proteins), dried *in vacuo* over P_2O_5 and restored to its original volume by the addition of distilled water. The washing procedure used at the end of these experiments was similar to that used for the *in vivo* preparation.

The *in vitro* production of $^{14}CO_2$ by abdominal nerve cords was measured in radioactive haemolymph and in ^{14}C-labelled glucose solutions using a procedure based on that of Villee & Hastings (1949). The apparatus used consisted of a microdiffusion cell with a ground-glass lid containing two 8·0 mm. diameter glass cups; the first held the radioactive solution and the nerve cord and the second 0·2 ml. of 5·0% NaOH with a small roll of filter-paper. The apparatus was gassed with a stream of oxygen for 10 min. before the commencement of each experiment. The radioactivity absorbed by the NaOH was subsequently measured with a scintillation counter.

The radioactivity extracted from the nerve cords was assayed using a thinwindowed G.M. tube (Mullard MX/123) or, for low activities, a Panax liquid scintillation counter (SC/LP). With this latter instrument the radioactive sample (*c.* 0·2 ml.) was dissolved in 3·0 ml. of a dioxane liquid phosphor. The lead castle of the scintillation counter was cooled with tap water and at an operating voltage of 1200 V. the instrument showed a background activity of about 30 counts/min.

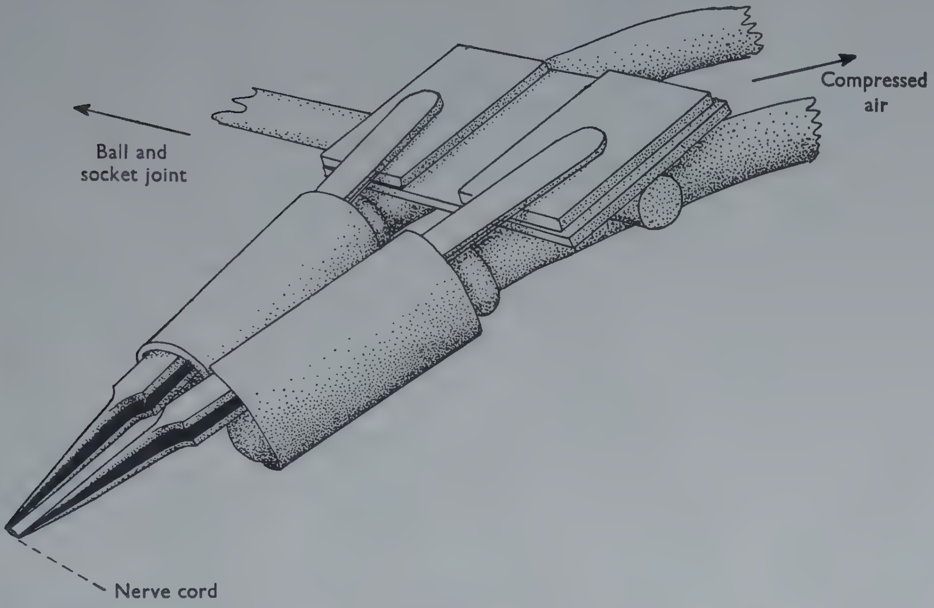

Fig. 1. Apparatus used to hold single connectives, ganglia and whole nerve cords. The automatically operated forceps were mounted on greased ground-glass plates.

The separation and identification of compounds present in the nerve cord and haemolymph was achieved by paper chromatography. The freshly collected haemolymph was precipitated with 2 vol. of ethanol, dried *in vacuo* over P_2O_5 and then restored to twice its original volume with 60% ethanol. Whole nerve cords were removed from the insects, washed for 90 sec. in ice-cold saline, plunged into liquid nitrogen and then ground up in either N formic acid in 50% ethanol, dimethyl formamide or 5% trichloro-acetic acid. The trichloro-acetic acid was subsequently removed from the solution by washing seven times with ether. Essentially similar results were obtained with all three solutions. For the detection of compounds by conventional chemical sprays ten nerve cords were extracted for each chromatogram; with radioactive nerve cords only one was necessary per chromatogram. Successive 3·0 μl. aliquots of the extracts were transferred to Whatman no. 1 filter-paper for separation by descending chromatography. The solvents used for one-dimensional chromatography were: phenol-water, *n*-propanol/ethyl acetate/water (Baar & Bull, 1953), 70% *n*-propanol (Chen, 1958), *n*-propanol/ammonia/water (Hanes & Isherwood, 1949), butanol/acetic acid/water (Synge, 1951) and ethyl acetate/acetic acid/water (Jermyn & Isherwood, 1949). Two-dimensional chromatograms were developed with 70% *n*-propanol followed by water-saturated phenol (Chen, 1958) and *n*-butanol/acetic acid/water (74/19/50) followed by phenol (Fowden & Steward, 1957). Ninhydrin-positive substances were detected by the ninhydrin-cobalt chloride spray of Wiggins & Williams (1952); reducing substances by the silver nitrate method of Trevelyan, Proctor & Harrison (1950); non-reducing carbohydrates by the sodium-metaperiodate spray of Evans & Dethier (1957) followed by silver nitrate; phosphorus compounds by the acid-molybdate method of Hanes & Isherwood (1949); and non-volatile aliphatic acids with the bromo-phenol blue spray of Lugg & Overell (1947). The concentrations of trehalose and glucose in the haemolymph were estimated by the method of Dimler, Schaefer, Wise & Rist (1952). Hydrolysis of oligo- and polysaccharides was effected by boiling in 3 N-HCl in a sealed tube for 3 hr., the products being re-run on fresh chromatograms.

Assay of radioactivity on chromatograms was carried out by moving the paper strips across a 1·0 cm. wide slit above a GM tube, successive counts being made until the whole paper was completed. With chromatograms of low activity a scintillation technique was employed in which the ^{14}C was eluted from 1·0 cm. wide pieces of the chromatogram and counted in the liquid phosphor. In the technique which was finally employed each 1·0 cm. wide piece of chromatogram paper was placed in the 30·0 ml. glass container and soaked with 0·2 ml. of distilled water. After 1 hr. 3·0 ml. of dioxane phosphor were added and the paper was removed, care being taken to drain the liquid from the paper in doing so. With radioactive glycogen it was necessary to soak the chromatogram strip overnight or to extract the radioactivity by boiling in a sealed glass tube.

The insects were reared and all experiments were performed at a temperature of 28·0 ± 1·0° C.

RESULTS

Haemolymph carbohydrates

Chromatographic analysis of the haemolymph revealed the presence of traces of glucose together with massive amounts of a non-reducing substance, subsequently identified as trehalose. The identification of this compound was based on the fact that acid hydrolysis yielded only glucose molecules and on the very close similarity of its position to that of authentic trehalose on chromatograms developed with four different solvent systems.

The concentrations of these two carbohydrates in the haemolymph of adult male cockroaches are summarized in Table 1. These results show a very high concentration of the disaccharide as compared with the low and extremely variable glucose content.

Experiments were performed to determine the metabolic fate of ^{14}C-labelled glucose introduced into the haemolymph. To do this 10·0 μl. of a 48·6 mM./l. solution were injected into individual cockroaches from which haemolymph samples were extracted after varying experimental periods. The radioactivity in the haemolymph was separated on paper chromatograms, some typical results being illustrated in Fig. 2. It was found that in all cases the injected glucose was rapidly converted to trehalose, which accumulated in the haemolymph, only very small amounts of glucose remaining in equilibrium with the trehalose.

Table 1. *The concentrations of trehalose and glucose in the haemolymph of adult male cockroaches*

Serial	Trehalose (mg./100 ml.)	Glucose (mg./100 ml.)
1	999·3	18·0
2	1517·1	27·0
3	1196·7	18·0
4	1281·2	58·7
5	1273·8	39·9
6	1606·9	72·1
7	1815·5	43·2
Mean	1395·9	39·5

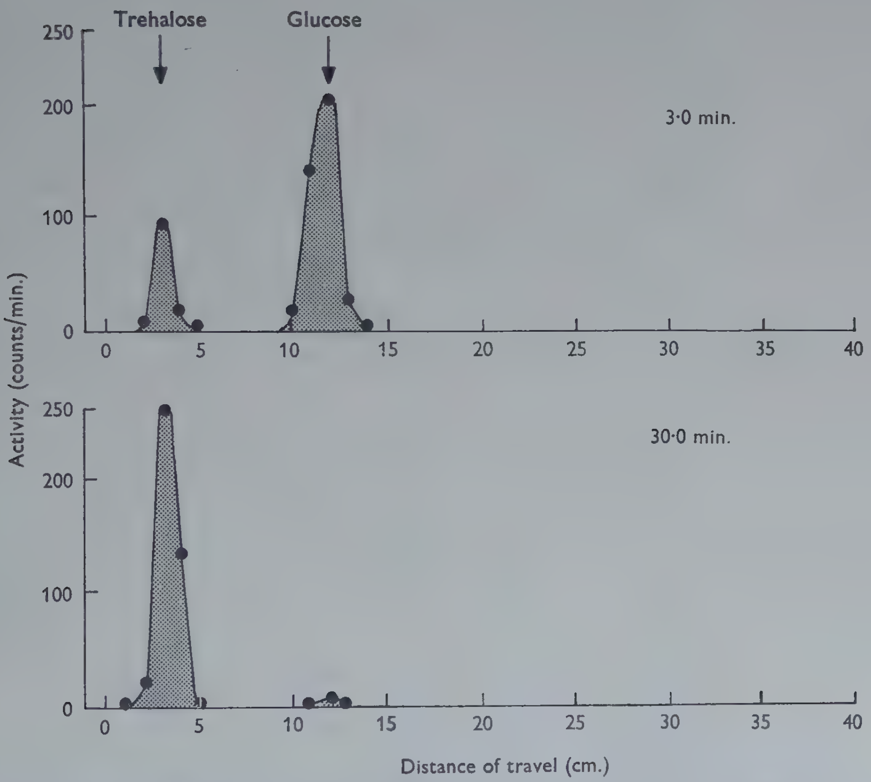

Fig. 2. Illustrating the distribution on chromatograms, developed with ethyl acetate/acetic acid/water, of radioactivity from the haemolymph of insects 3·0 and 30·0 min. after the injection of ^{14}C-labelled glucose.

Washing and efflux experiments

In order to determine the amounts of ^{14}C-labelled compounds contained in the experimental abdominal nerve cords it was necessary to devise some methods by which the surface radioactivity, from the haemolymph, could be separated from that contained within the nerve cords. A series of experiments was, therefore performed in which radioactive abdominal nerve cords were removed from injected cockroaches and washed in successive 0·2 ml. amounts of Hoyle's saline. At the end of the series of washings the radioactivity remaining in the cords was extracted in the usual way. From the activities of the washings and the final extraction it was possible to construct graphs showing the amount of ^{14}C remaining associated with the nerve cords throughout the experiment. Such a graph is illustrated in Fig. 3 and shows the decline in radioactivity associated with a nerve cord taken from an insect injected 1 hr. previously with 10·0 μl. labelled glucose solution. The curve in Fig. 3*a* when plotted on a logarithmic scale with respect to time (Fig. 3*b*) can

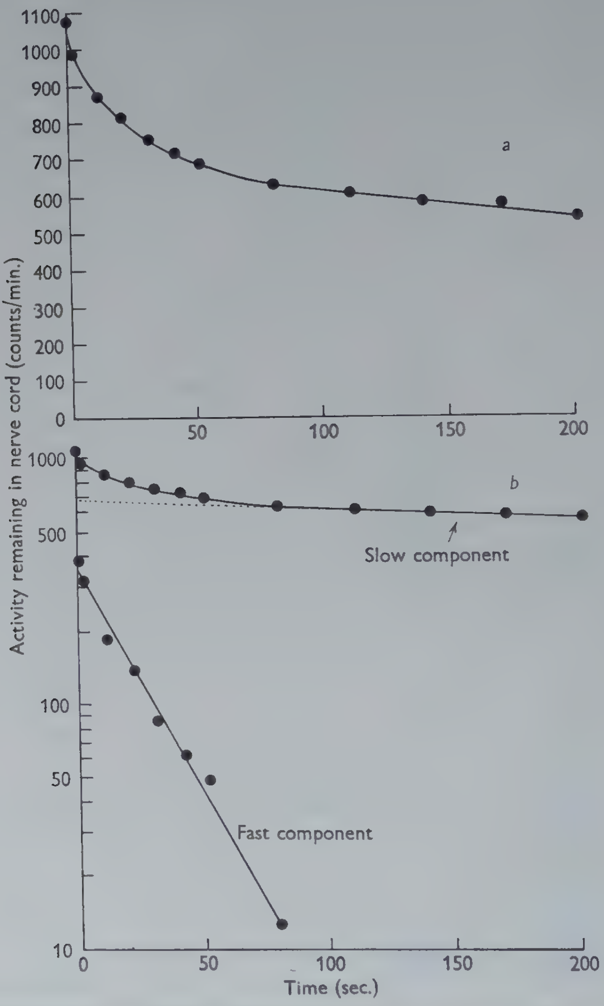

Fig. 3. (*a*) The radioactivity associated with the abdominal nerve cord after washing in successive volumes of saline. The insect from which this nerve cord was removed had been injected with 10·0 μl. of ^{14}C-labelled glucose 1 hr. previously. (*b*) The data plotted on a logarithmic scale with respect to time, showing the resolution into two components.

be resolved into two components: a rapid initial decline with a half time of 19 sec., and a second slow fall with a half time of about 580 sec. This effect is essentially similar to that obtained for the efflux of tritiated water from isolated lobster nerves (Nevis, 1958).

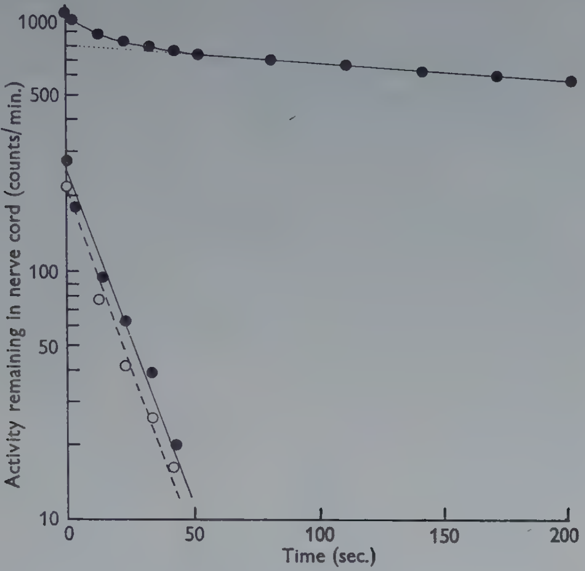

Fig. 4. The decline in radioactivity during washing of isolated nerve cords previously soaked in ^{14}C-labelled glucose for 1 hr. and for 1 sec. The data for the nerve cords soaked for 1 hr. have been resolved into the two components; those soaked for 1 sec. showed only a single rapid component.

In some further experiments isolated abdominal nerve cords were soaked in a 4·9 mM./l. ^{14}C-glucose solution for a period of 1 hr. and the subsequent efflux of radioactivity on washing in a succession of 0·2 ml. amounts of saline was recorded. These results were compared with the efflux obtained from isolated cords which were submerged in the radioactive solution for a period of only 1 sec. before washing in the usual way. Both sets of results are summarized in Fig. 4, from which it will be seen that the efflux from the nerve cord soaked for 1 hr. could be resolved into two components, which were similar to those for cords taken from intact insects (Fig. 3). The efflux from the cord exposed for 1 sec. showed, however, only a single rapid component which paralleled that for the cords soaked for 1 hr. It seems most reasonable to conclude from these results that the rapid component represented the washing of ^{14}C-labelled material from the surface and the slow one the loss from the interior of the nerve cord. Thus to determine the total activity of the ^{14}C-labelled components within the nerve cord it was only necessary to extrapolate the slow component back to zero time. In all experiments the total radioactivities in the nerve cords have been obtained and calculated in this way, the activity being expressed in terms of that contained in unit volume of nerve cord water.

The accumulation of radioactive material in the abdominal nerve cord

The increase in radioactivity in the nerve cord was studied following the injection of 10·0 μl. of the ^{14}C-labelled glucose solution into the haemolymph. The individual cockroaches used in these experiments showed great variability in haemolymph volume, with a resulting variation of injected radioactivity expressed per unit volume of haemolymph. To minimize this source of variation all results for the accumulation of ^{14}C-labelled material in the nerve cords are expressed as the ratio: activity in nerve cord/activity in haemolymph.

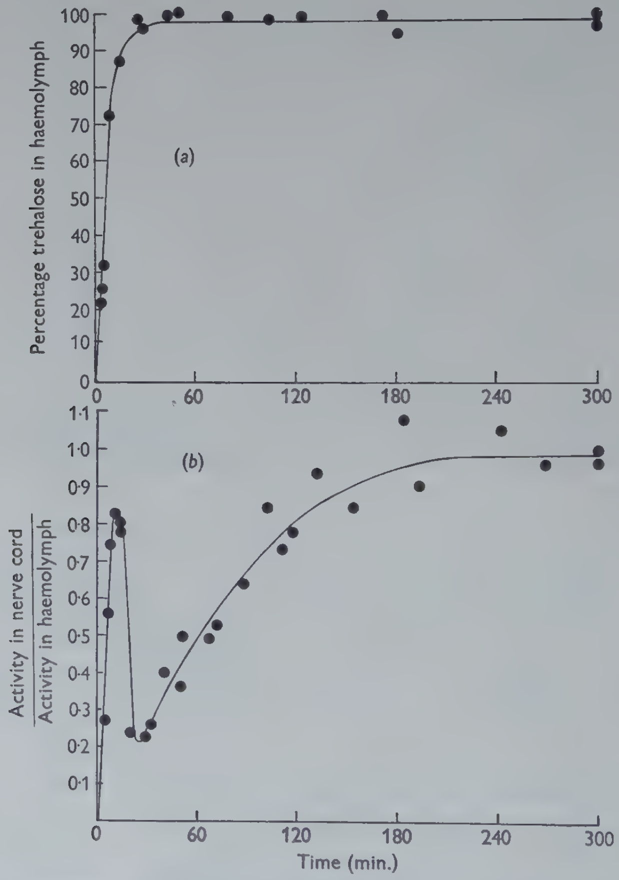

Fig. 5. The rate of conversion of injected ^{14}C-labelled glucose to trehalose, which accumulated in the haemolymph (*a*), compared with the entry of radioactivity into the nerve cord expressed as the ratio: activity in nerve cord/activity in haemolymph (*b*).

The entry of radioactive material into the nerve cord following the injection of ^{14}C-labelled glucose is illustrated in Fig. 5*b*, where it is also compared with the rate of conversion of glucose to trehalose in the haemolymph. It will be seen that there was an initial rapid rise of radioactivity in the abdominal nerve cord relative to that in the haemolymph. This activity reached a peak after about 10 min. and was followed by a fall, to a ratio of about 0·2 after 30 min. During this period the glucose was converted to trehalose, which accumulated in the haemolymph, reaching an equilibrium by about 30 min. After this the activity in the nerve cord rose again rather more slowly to a level close to that in the haemolymph.

The significance of the form of this graph will be discussed in a later section, it is only necessary here to mention that the second build-up of radioactivity is of interest because it represents the accumulation of ^{14}C originating from trehalose which is in equilibrium with the small amount of glucose in the haemolymph.

A further series of experiments was performed in which the radioactivities in single ganglia and connectives were compared. Fig. 6 illustrates the increase in radioactivity, relative to that in the haemolymph, in the last abdominal ganglion and in the connective between the fourth and fifth abdominal ganglia. The form of this graph is essentially similar to that obtained for the whole nerve cord and it is clear that the radioactivity appeared at similar rates in the different parts of the central nervous system.

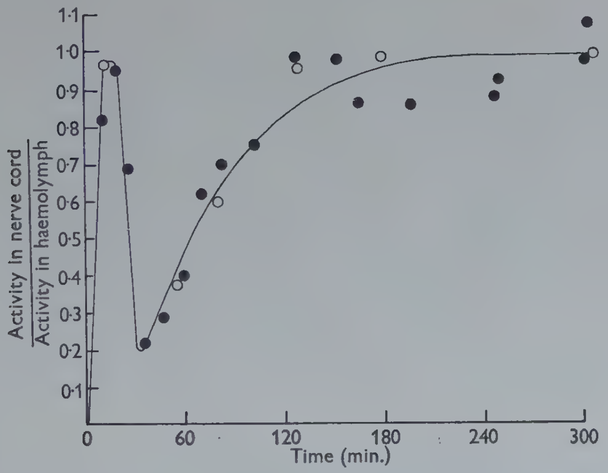

Fig. 6. The rate of accumulation of radioactivity in the last abdominal ganglia and in the connectives between the 4th and 5th ganglia expressed as the ratio: activity in nerve cord/activity in haemolymph. ●, Connective; ○, terminal abdominal ganglia.

CO_2 *production by the isolated nerve cord*

The above experiments which recorded the accumulation of radioactivity within the abdominal nerve cord do not necessarily give an accurate picture of the movement of ^{14}C-labelled compounds into the nerve cord, for no account could be taken with the intact insect of the $^{14}CO_2$ produced by the nervous tissue. Experiments were, therefore, performed to measure the rate of $^{14}CO_2$ production using isolated, ligatured nerve cords in the radioactive haemolymph solution. The $^{14}CO_2$ evolved was absorbed in 5·0% NaOH and its activity assayed using the scintillation counter. The $^{14}CO_2$ production was also measured in a ^{14}C-glucose solution (75·99 mM./l.)

of concentration equivalent to the combined trehalose and glucose content of the haemolymph. The results are tabulated in Table 2, from which it will be seen that the $^{14}CO_2$ produced *in vitro* represented only a very small part of the total radioactivity contained within the nerve cord.

Table 2. *The $^{14}CO_2$ production of isolated abdominal nerve cords after a period of* 1 *hr.*

Solution	Activity in nerve cord (counts/min.)	Activity as $^{14}CO_2$ (counts/min.)	$^{14}CO_2$ production (%)	
Radioactive haemolymph	2,992·0	27·0	0·90	1·04 %
	3,357·4	42·1	1·25	
	3,689·8	49·1	1·30	
	3,962·8	44·4	1·11	
	1,949·8	12·5	0·64	
Glucose (75·99 mM./l.)	6,095·4	117·3	1·92	1·02 %
	12,078·0	140·0	1·16	
	21,281·0	360·3	1·69	
	19,588·0	126·3	0·64	
	25,236·0	203·3	0·80	

Sugar fluxes in the nerve cord

At this stage it was essential to obtain a quantitative estimate of the entry of ^{14}C-labelled sugars into the nerve cord. It was also necessary to discover the proportion of the radioactivity originating from the trehalose and from the small amount of glucose in equilibrium with the disaccharide in the haemolymph. Fortunately the apparently slow rate of $^{14}CO_2$ production by the nerve cord enabled the influx of the sugars to be calculated, with only negligible error, according to the equation given by Croghan (1958) for ion fluxes:

$$m.t = C_i \log_e z/(z-y),$$

where m = total sugar influx, expressed as mM. glucose units/litre of nerve cord water/min.;
$z = C_i x/C_0$ (i.e. the activity in the nerve cord when exchange is complete);
x = activity in haemolymph (counts/unit volume);
y = activity in nerve cord (counts/unit volume);
C_i = concentration of ^{14}C-labelled compounds in the nerve cord, expressed as equivalent glucose units (75·19 mM./l.);
C_0 = concentration of ^{14}C-labelled trehalose and glucose in the haemolymph, expressed as glucose units (75·99 mM./l.).

(In this section all trehalose concentrations are expressed as equivalent glucose concentrations in mM./l.)

The fluxes of the ^{14}C-labelled sugars were compared as between:

(1) Intact insects, in which the nerve cord radioactivity originated as trehalose and glucose in the haemolymph.

(2) Isolated preparations, in which the trehalose and glucose were labelled with ^{14}C in the haemolymph solution.

(3) Isolated preparations, in which only the glucose (2·19 mM./l.) was labelled with ^{14}C, the trehalose being non-radioactive.

The data for the flux in the intact insect are taken from Fig. 5 and represent the entry of radioactivity (after 30 min.) when the injected glucose had come into equilibrium with the trehalose. These values have been corrected to zero time for this calculation.

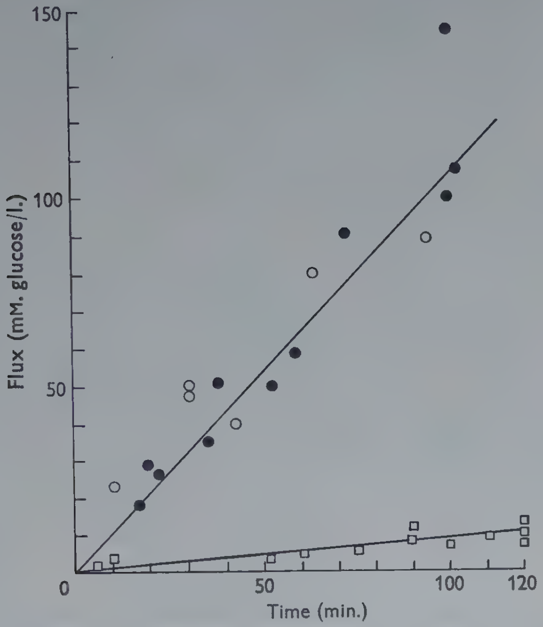

Fig. 7. The calculated influx of radioactivity originating as: (i) trehalose and glucose in the haemolymph of intact insects (closed circles); (ii) as trehalose and glucose for connectives isolated in radioactive haemolymph (open circles); (iii) for isolated connectives in saline in which only the glucose (2·19 mM./l.) was labelled with ^{14}C, the trehalose being non-radioactive (open squares). The fluxes are expressed as equivalent mM. ^{14}C-labelled glucose/litre nerve cord water/min.

From Fig. 7 it will be seen that on plotting $2{\cdot}3\ C_i \log_{10}[z/(z-y)]$ against t approximately straight lines were obtained in all three cases. The flux in the intact preparation was, apparently, similar to that of the isolated connective immersed in the haemolymph solution, and was equivalent to 1·09 mM. glucose/l. nerve cord water/min. The influx when only glucose was labelled was approximately 0·08 mM./l. nerve cord water/min. Thus it seems that the radioactivity originating from the glucose accounted for about 7·3 % of the ^{14}C entering the abdominal nerve cord.

The metabolism of ^{14}C-labelled sugars in the nerve cord

In order to follow the metabolic fate of the sugars entering the central nervous system the radioactivity was extracted, and subsequently separated on paper chromatograms, from nerve cords of cockroaches injected 3 hr. previously with 10·0 μl. of ^{14}C-glucose solution. It was found that the extracted ^{14}C could be separated into at least seven peaks of radioactivity. It seemed likely that some of these radioactive peaks might represent ^{14}C incorporated in phosphorus compounds. The phosphorus spots detected on the chromatograms with the acid-molybdate spray did not, however, invariably correspond with these peaks of radioactivity, while ^{32}P injected as orthophosphate was not consistently associated with the ^{14}C. Negative results were obtained also in tests for the incorporation of the ^{14}C as lactic and pyruvic acids. Several of these zones of ^{14}C activity were, however, found to be associated with some ninhydrin-positive spots obtained from nerve cord extracts. These ninhydrin-positive substances present in the nerve cord were separated on two-dimensional chromatograms (Fig. 8) which revealed the presence of glycine, alanine, serine, aspartic acid, glutamic acid and glutamine. These substances, together with glucose and trehalose, were run with nerve cord extracts on chromatograms developed in four different solvent systems (Fig. 9). From these chromatograms it was possible to identify the major radioactive components as: aspartic acid, glutamic acid, glutamine, occasionally small amounts of alanine, trehalose and glucose. The peak of radioactivity at the base line ($R_f = 0$) was found to be water-soluble, alcohol-insoluble and to yield only glucose on acid hydrolysis (Fig. 9*e*), indicating the presence of ^{14}C-labelled glycogen in the nerve cords. None of the activity was found to be associated with added γ-amino-butyric acid.

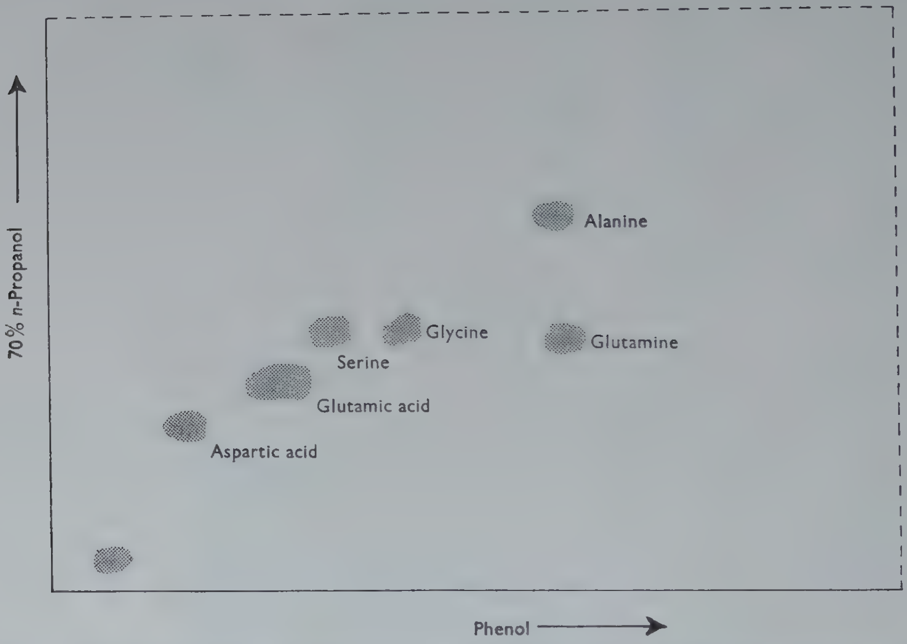

Fig. 8. The distribution of ninhydrin-positive substances extracted from ten abdominal nerve cords on two-dimensional chromatograms developed with 70 % propanol followed by water-saturated phenol.

The proportions of the various radioactive substances accumulating within the nerve cord following the injection of ^{14}C-labelled glucose into the haemolymph

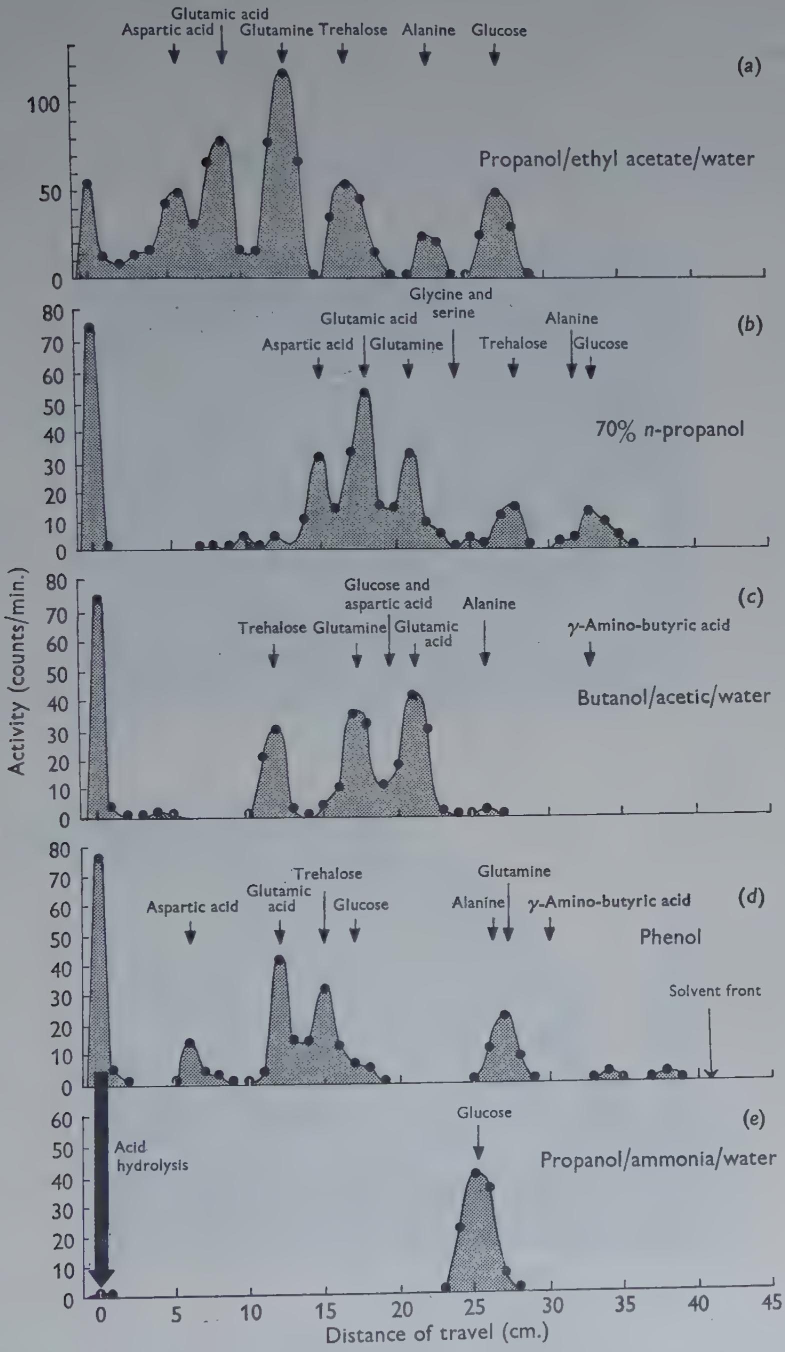

Fig. 9. The distribution of radioactivity extracted from abdominal nerve cords 3 hr. after injection of ^{14}C-labelled glucose into the haemolymph developed in four different solvent systems (*a–d*). A chromatogram of the radioactivity from the peak at the base-line (i.e. $R_f = 0$) after acid hydrolysis is illustrated in (*a*).

are summarized in Fig. 10*a*. This data, together with that illustrated in Fig. 5, has been used to construct a diagram showing the concentrations of these compounds in the nerve cord relative to that of the labelled sugars in the haemolymph (Fig. 10*b*).

The *in vitro* metabolism of ^{14}C-labelled glucose was studied by immersing isolated nerve cords in 2·19 mM./l. glucose saline solution containing non-radioactive

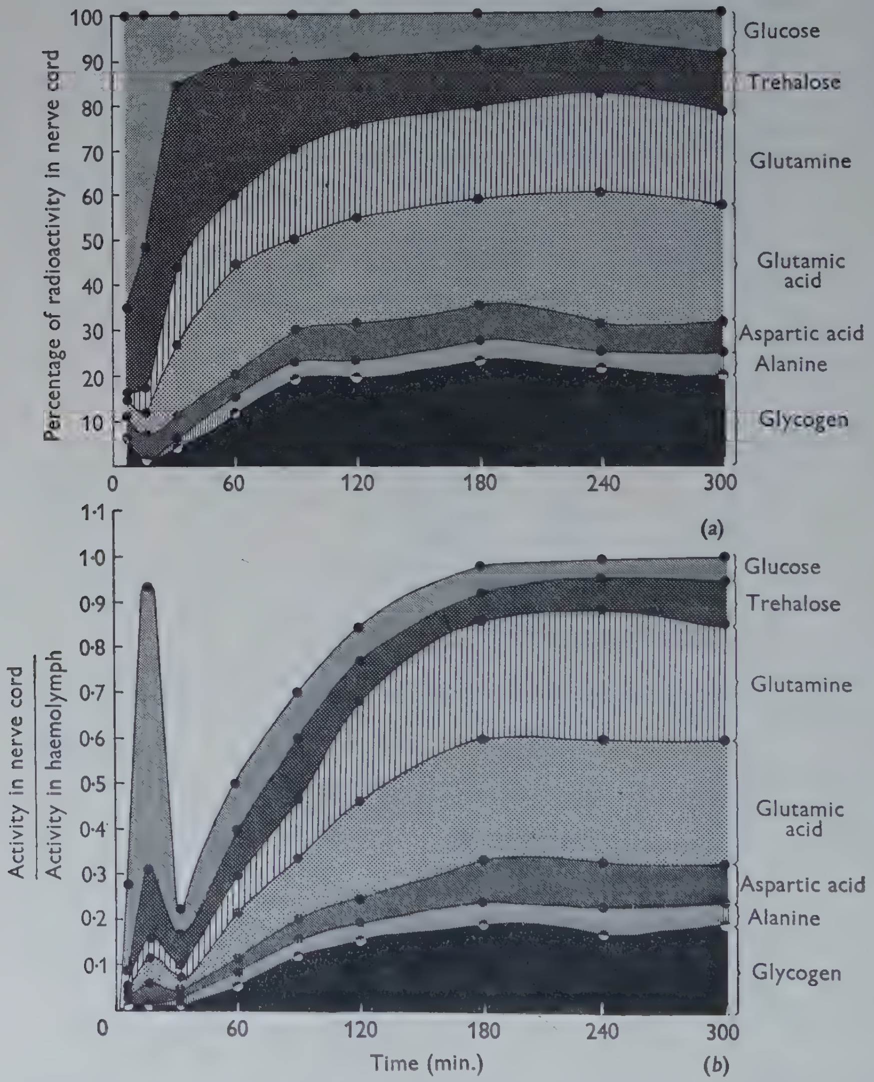

Fig. 10. (*a*) The proportions of the various ^{14}C-labelled compounds which accumulated in the nerve cords following injection of ^{14}C-labelled glucose into the haemolymph. (*b*) The estimated concentrations of the ^{14}C-labelled compounds in the nerve cords (expressed as the ratio: activity in nerve cord/activity in haemolymph) calculated from the data given in (*a*) and Fig. 5 (*b*).

trehalose (36·90 mM./l.). After 2 hr. the preparations were washed and the extracted radioactivity was run on chromatograms developed with four different solvent systems (Fig. 11). In these preparations only occasional traces of aspartic acid, glutamic acid, glutamine and trehalose were found, but alanine accumulated in relatively large amounts together with massive concentrations of glucose. The appreciable amounts of radioactivity remaining at the base line yielded only glucose on acid hydrolysis and were presumed to be glycogen.

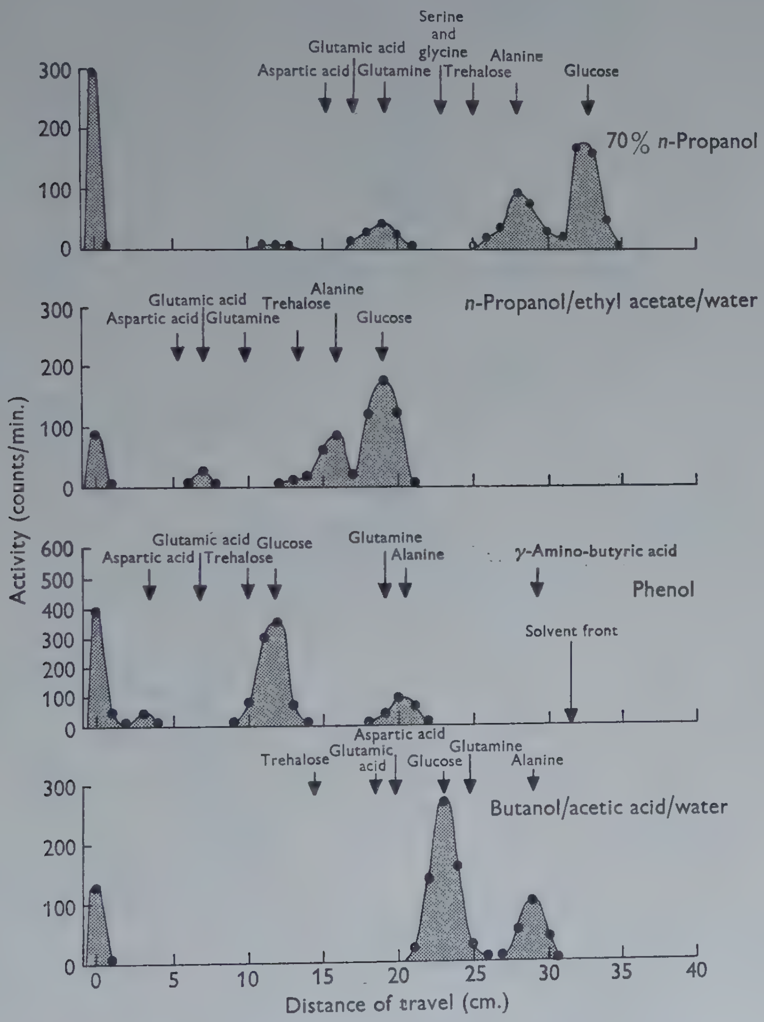

Fig. 11. The distribution of radioactivity extracted from nerve cords isolated in 2·19 mM./l. ^{14}C-labelled glucose and non-radioactive trehalose developed in four different solvent systems.

Some further experiments using isolated preparations were performed in which the nerve cords were immersed in the radioactive haemolymph solution or in a saline solution containing labelled glucose in equivalent concentration to that of the trehalose and glucose in the haemolymph (75·99 mM./l.). In both cases substantial amounts of alanine were found to have accumulated with the isolated nerve cords (Fig. 12). In the cords isolated in the radioactive haemolymph glucose, together with some reduced amounts of glutamic acid, accumulated in addition to the trehalose; the cords placed in the high-glucose solution showed concentrations of alanine and glycogen in addition to the large amounts of glucose present.

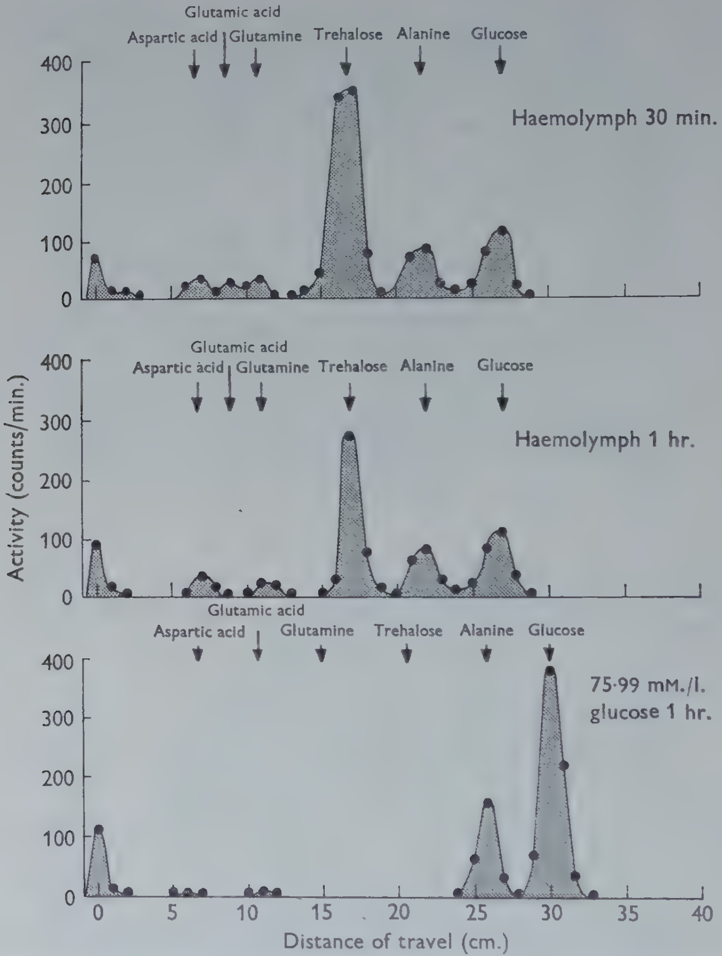

Fig. 12. Chromatograms of radioactivity extracted from nerve cords isolated in radioactive haemolymph and in a solution of 75·99 mM./l. ^{14}C-labelled glucose. These chromatograms were developed in ethyl acetate/acetic acid/water.

DISCUSSION

The cockroach can now be added to the number of insects which have been found to possess haemolymph containing appreciable amounts of the disaccharide trehalose (Wyatt & Kalf, 1956; Howden & Kilby, 1956; Evans & Dethier, 1957). The concentration of 1·39% in the haemolymph of this insect is of the same order as that found in the locust, *Schistocerca gregaria* (Howden & Kilby, 1956; Treherne, 1958*b*) and the blowfly, *Phormia regina* (Evans & Dethier, 1957). The rapid conversion of glucose to trehalose, which accumulates in the haemolymph, has also been previously demonstrated in the locust (Treherne, 1958*a*, *b*), the actual conversion apparently taking place in the fat body of this insect (Candy & Kilby, 1959). In the cockroach, as in the locust, a small amount of glucose (*c*. 39·5 mg./100 ml.) remained in equilibrium with the trehalose in the haemolymph.

The movement of ^{14}C into the abdominal nerve cord after injection of a small amount of radioactive glucose into the haemolymph followed a rather complex course (Figs. 5, 6). Immediately after the injection of labelled glucose there was a very rapid increase in radioactivity within the nerve cord which, after about 10 min., fell sharply to about a fifth of the activity in the haemolymph. After approximately 30 min. the activity commenced to rise again, this time more slowly, to a level similar to that in the haemolymph. When considering these results it should be borne in mind that the specific activities of the haemolymph carbohydrates were changing rapidly during the first 30 min. of the experiments. The initial steep rise in activity represented the movement of relatively few glucose molecules, of very high specific activity, into the nerve cord. During this period the glucose was rapidly converted to trehalose which accumulated in the haemolymph, the whole system approaching equilibrium after about 30 min. The second slow increase in radioactivity thus represented the entry of ^{14}C originating as trehalose or as the small amount of glucose remaining in the haemolymph. This interpretation is supported by the subsequent chromatographic analysis when it was found that during the first part of the experiment the ^{14}C was largely incorporated as glucose in the nerve cord. Thus the initial rise and fall in activity can be regarded as an experimental artifact, the true movement of sugars into the abdominal nerve cord being represented by the second uptake curve when the ^{14}C-labelled trehalose was in effective equilibrium with the small amount of glucose in the haemolymph.

The appearance of radioactivity in different parts of the nerve cord was shown to occur at closely similar rates and to parallel the uptake of the ^{14}C-labelled sugars by the whole system. Such a result could be obtained either by the sugars passing through the different surfaces at similar rates or, if the passage through these surfaces is unequal, by a rapid movement of ^{14}C-labelled compounds within the central nervous system.

The influx of ^{14}C originating from the labelled trehalose and glucose in the haemolymph was calculated to be equivalent to about 1·09 mM. glucose/l. of nerve cord water/min. This apparently rapid flux could be brought about by an entry of trehalose or by a relatively rapid movement of molecules from the small amount of glucose in the haemolymph. Some light was thrown on the process by the measured influx in the isolated preparation when only the glucose was labelled with ^{14}C, the trehalose being non-radioactive. Here the apparent flux was equivalent to 0·08 mM./l. nerve cord water/min., which suggests that only about 7% of the activity in the nerve cord originated from the small glucose pool in the haemolymph.

This means that one molecule of glucose passed into the nerve cord for approximately every seven molecules of trehalose. Trehalose molecules are, however, seventeen times more concentrated than those of glucose in the haemolymph, which implies that the individual glucose molecules were in fact passing into the nerve cord at about 2·5 times the rate of the trehalose molecules. The diffusion constant of glucose through water ($0{\cdot}68 \times 10^{-5}$ cm.^{2}/sec. at 25° C., *International Critical Tables*) is, on the other hand, only 1·2 times that of a disaccharide (sucrose $-0{\cdot}55 \times 10^{-5}$ cm.^{2}/sec.). Such apparent discrepancy between the rates of passage of these two sugars into the nerve cord and their free diffusion constants could be brought about, for example, by some sort of transport of glucose, or by the slowing down of trehalose due to steric or frictional hindrance of the larger disaccharide molecule in diffusing through some kind of pore membrane. It would be unwise, however, to press these conclusions too far at this stage. The glucose influx was calculated from data obtained with the isolated preparation in which these processes might be altered in some way. Against this should be set the fact that the influx from the radioactive haemolymph was similar in the intact insect, although this result is further complicated by the changed metabolism of the ^{14}C-labelled compounds within the isolated nerve cord.

The prompt appearance of ^{14}C, supplied as trehalose and glucose, in the carbon skeletons of aspartic and glutamic acids *in vivo* and in appreciable amounts of alanine *in vitro* represents circumstantial evidence for the presence of the Krebs tricarboxylic acid cycle enzymes in the central nervous system of this insect. The identification of glucose-6-phosphate and phosphoglyceric acid in the cockroach nerve cord (Heslop & Ray, 1958) suggests that the carbohydrate metabolism is linked to the tricarboxylic acid cycle by the conventional glycolytic pathway. Thus alanine could be produced by the amination of the resulting pyruvic acid and aspartic acid by the amination of oxalacetic acid derived from the pyruvic acid. Glutamic acid formation would result from the amination of α-ketoglutaric acid synthesized by the enzymes of the tricarboxylic acid cycle.

More than half of the administered ^{14}C was found to be incorporated as glutamic acid and glutamine in the intact nerve cord, suggesting that the very reactive amino acid occupies a central position in the metabolism of the central nervous system in this insect. The linkage with glutamine is of especial importance for this substance must function as an important reservoir of amino-nitrogen in the nervous tissue. The high concentration of these two substances, together with the relatively low carbohydrate content, is essentially similar to the state of affairs in the mammalian brain tissue (cf. McIlwain, 1959). It is, therefore, of interest to note that in isolated rat and guinea-pig cerebral cortex administration of glutamic acid led to respiratory rates higher than those induced by glucose (Weil-Malherbe, 1936).

The presence of glycogen has been previously demonstrated histochemically in the central nervous system of the mosquito larva (Wigglesworth, 1942), where it occurs in granular deposits and vacuoles, and in the cockroach nerve cord (Wigglesworth, 1960). The synthesis of glycogen within the nervous systems of these insects was demonstrated by following its accumulation after feeding starved individuals with various carbohydrates. A linkage of carbohydrate metabolism and glycogen synthesis with the amino acid metabolism can also be inferred from the observations of Wigglesworth (1942) that the feeding of starved larvae with alanine and glutamic acid resulted in an accumulation of glycogen in the ganglia and connectives of the nerve cord.

Finally, it is relevant to compare the distribution of ^{14}C among the compounds in the cockroach nerve cord with that obtained for the mammalian central nervous system. Isolated rat-brain cortex slices can convert ^{14}C-glucose into labelled glutamic acid, aspartic acid, alanine and glutamine (Kini & Quastel, 1959) as in the cockroach. The rat nervous tissue differed from that of the cockroach in the formation of appreciable amounts of γ-amino-butyric acid, a substance which was not detected among the labelled compounds in the insect nerve cord. Some other major differences in the metabolism of the ^{14}C in the nervous system of these two animals were an apparent absence of glycogen formation, an accumulation of large amounts of lactic acid and a relatively high rate of $^{14}CO_2$ production in rat-brain slices (Beloff-Chain, Catanzaro, Chain, Masi & Pocchiari 1955). The possibility cannot of course be eliminated that some of the apparent differences in the metabolism of ^{14}C may have been due to the different techniques employed, for the rat brain was studied in isolation and the cockroach nerve cord *in vivo*. It should be mentioned in this respect that in the present investigation the *in vitro* preparations did not accumulate the various ^{14}C-labelled compounds in the same proportion as the nerve cords of an intact insect.

SUMMARY

1. ^{14}C-labelled glucose injected into the cockroach was found to be rapidly converted to trehalose, only small amounts remaining in equilibrium with the disaccharide in the haemolymph. The entry of these sugars into the cockroach central nervous system was studied by following the increase in radioactivity within the abdominal nerve cord after the injection of radioactive glucose into the haemolymph.

2. The levels of radioactivity increased at closely similar rates in different parts of the abdominal nerve cord.

3. The influx of sugars into the nerve cord was calculated to be equivalent to 1·09 mM. glucose/l. of nerve cord water/min.

4. The greater part of the ^{14}C entering the nerve cord originated from the trehalose, only about 7% being derived from the small amount of glucose in the haemolymph. The movement of the relatively small number of glucose molecules into the nerve cord occurred, nevertheless, at approximately 2·5 times the rate of the larger trehalose molecules.

5. Chromatographic analysis revealed that more than half of the absorbed ^{14}C was incorporated as glutamic acid and glutamine in the nerve cord. Smaller amounts of glycogen, trehalose, glucose, aspartic acid and occasional traces of alanine were found. In the isolated nerve cord substantial amounts of alanine

accumulated, the formation of the other amino acids being reduced. $^{14}CO_2$ production *in vitro* was found, after 1 hr., to represent only about 1% of the total activity within the nerve cord.

6. The results demonstrate a linkage of carbohydrate and amino acid metabolism and represent circumstantial evidence for the presence of the tricarboxylic acid cycle enzymes in the central nervous system of this insect.

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THE ABSORPTION OF SODIUM IONS BY THE CRAYFISH, *ASTACUS PALLIPES* LEREBOULLET

II. THE EFFECT OF THE EXTERNAL ANION

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INTRODUCTION

In the first paper of this series (Shaw, 1959) it was shown that sodium balance in the freshwater crayfish, *Astacus pallipes*, was maintained as a result of the opposing effects of the external and internal sodium concentration on the rate of sodium uptake. This self-balancing system was found to operate under experimental conditions where the external medium consisted of pure sodium chloride solutions. In the normal environment of the crayfish sodium and chloride ions, although present, represent only a fraction of the total ionic content of the water. Natural fresh water has a much more complex ionic composition in which divalent cations, such as calcium and magnesium, and anions such as bicarbonate and sulphate, are quantitatively important. In this paper the effect of the nature of the anion on the rate of uptake of sodium ions is considered.

The classical studies of Krogh (1937*a*, *b*, 1938, 1939) on the net uptake of sodium by certain freshwater animals which had been previously salt-depleted, showed that sodium ions could be taken up from a variety of sodium salts. In *Eriocheir* and the frog he found that net sodium absorption occurred from solutions of NaCl, $NaHCO_3$ and Na_2SO_4 and in the goldfish, *Carassius auratus*, from solutions of NaCl, NaBr, $NaHCO_3$ and $NaNO_3$. Krogh's results showed that the presence of chloride was not necessary for sodium absorption. He did not, however, record the rate of sodium uptake from the different solutions so that the influence of the nature of the anion on sodium transport could not be determined. He also demonstrated that chloride could be absorbed by these animals in the absence of sodium and he stressed the independent nature of the mechanisms for the uptake of the two ions.

The detailed investigations by Ussing and his associates (Ussing, 1949; Ussing & Zerahn, 1951; Koefoed-Johnsen, Ussing & Zerahn, 1952; Ussing, 1954) on the isolated frog skin contradicted this view in certain respects. They demonstrated conclusively that the transport of sodium ions across the isolated frog skin was an active process moving the ions against an electrochemical gradient, but a thorough study of the movement of chloride revealed that this ion was moving passively as a result of the electric field established by the transport of sodium ions (Koefoed-Johnsen, Levi & Ussing, 1952; Linderholm, 1952). An independent chloride uptake system was not demonstrated, although active secretion of chloride outwards was demonstrated after the action of adrenaline (Koefoed-Johnsen, Ussing & Zerahn, 1952).

The work of Jørgensen, Levi & Zerahn (1954) on intact frogs helped to throw some light on the divergence between the results of Krogh and Ussing. Measurements of the influx of sodium and chloride ions in the intact frog, together with measurements of the potential difference across the skin *in situ* supported the correctness of Krogh's original observations. Jørgensen *et al.* found that the influxes of the two ions could be very different from each other and that active transport of chloride could take place. They also found that in frogs deficient in sodium but not chloride, sodium influx was consistently greater than the chloride influx, thus indicating that the rate of sodium transport did not depend on the rate of chloride movement.

These experiments are of the greatest importance since they show that although studies on the isolated frog skin are extremely valuable for the analysis of many of the properties of a sodium-transporting system, an isolated preparation such as this may lose altogether some of the important characteristics which it displays in the living animal. This fact must always be borne in mind when attempting to make use of the results derived from the study of such preparations in interpreting the behaviour of the normal animal.

In cases where the uptake of sodium ions by the animal exceeds the uptake of the accompanying anion then the excess positive ion uptake must be balanced by the excretion of an equal number of positive ions of another kind. The nature of this exchange was investigated by Krogh (1939) but the details of his experiments were not reported. He states that in the goldfish, 'When this absorption (of Na) is not accompanied by an approximately equivalent absorption of Cl^- it is sometimes compensated by a definite increase in the ammonia excretion through the gills', and in the frog absorption from sodium sulphate is accompanied by a definite exchange with ammonia; and, again, in *Eriocheir* he asserts that sodium from sodium sulphate and bicarbonate is exchanged for ammonia. From these statements it would appear that generally sodium uptake is accompanied by an increase in ammonia excretion but that there is not always a 1:1 exchange between the two.

The object of the work reported in this paper was to measure the influx and net uptake of sodium ions by the crayfish in the presence of various anions and to attempt to characterize the exchange process when the anion does not penetrate.

MATERIAL AND METHODS

Experiments were performed on the common British crayfish, *Astacus pallipes* Lereboullet, as previously described (Shaw, 1959).

Sodium influx was measured by means of the radioactive isotope ^{24}Na, as before, and in some of the earlier experiments the same technique was employed. For the more recent experiments an apparatus was designed which allowed a more continuous recording of the radioactivity of the external solution and, hence, a more rapid and accurate measurement of the influx. The apparatus is shown diagrammatically in Fig. 1. The animal chamber consisted of an inner Perspex box situated within an outer one through which water at constant temperature (12° C.) was circulated. The water in the animal chamber itself was circulated by means of an air-lift through narrow polythene tubing and past a flow-type Geiger counter (20th Century Electronics, Type FM 6 H). The counter was screened all round by at least 2 in. of lead and the animal chamber was far enough from the counter for the radioactive solution in the animal chamber not to increase the natural background of the counter significantly. The volume of the circulating fluid was between 200 and 250 ml. For each influx measurement about 1 μc. of ^{24}Na was introduced into the animal chamber together with sufficient non-active sodium salt to give the desired concentration. The loss of radioactivity from the water after the introduction of an animal into the chamber was measured either by taking counts at intervals of about 6 min. or by recording continuously by means of a recording ratemeter. The influx was calculated in the manner previously described.

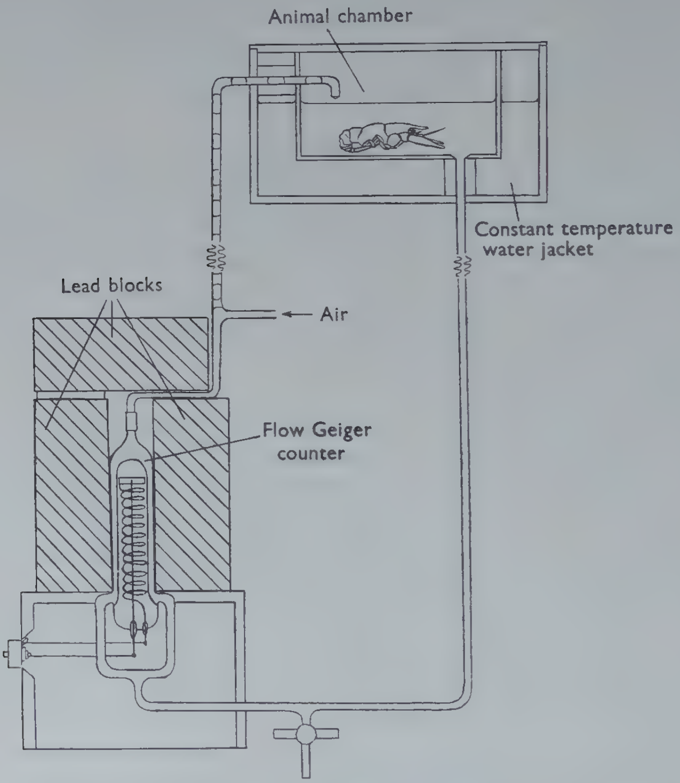

Fig. 1. The apparatus used for the continuous recording of the radioactivity in the external solution during sodium uptake.

Influx measurements were made on animals which had been previously salt-depleted by treatment with de-ionized water until the maximum influx (for a given external concentration) was attained. Under these conditions reproducible measurements of sodium influx in individual animals could often be obtained for several weeks.

Chemical measurements of sodium concentration were also made so that the net uptake of sodium could also be measured. Sodium taken up during an experiment was removed later by treatment with de-ionized water to prevent the adaptation of the influx to a lower level (Shaw, 1959).

Ammonia concentration in the external solution was measured colorimetrically using Nessler's reagent. Conductivities were measured with a dip-type cell and standard conductivity bridge.

RESULTS

(*a*) *The effect of the anion on the sodium influx*

Table 1 shows measurements of sodium influx from external solutions of sodium chloride, sulphate, nitrate and bicarbonate for six different animals. For two of the animals two different external concentrations were used for the same specimen. Generally the values of the influx at a given external concentration were virtually independent of the nature of the anion. In two animals (specimens 29 and 32) the influx from the chloride solutions was slightly greater than that from the sulphate. The difference, however, is probably not significant since the measurements were made on different days and variations of this order were to be expected. This is borne out by the fact that in certain cases the influx from sodium sulphate was measured and then potassium chloride was added after a period—the additional chloride did not lead to an increase in the sodium influx. It therefore appears that the nature of the anion has little or no effect on the sodium influx.

Table 1. *The effect of the nature of the anion on the sodium influx from solutions of sodium salts*

Specimen no.	External sodium concn. (m.equiv./l.)	Sodium influx (μM./hr.)			
		Chloride	Sulphate	Nitrate	Bicarbonate
25	0·15	5·9	6·5	—	—
27	0·55	10·1	10·1	—	16·6, 10·8
27	1·05	15·0	—	14·6	—
29	0·55	—	12·0, 12·8	12·8	—
29	1·10	17·0	14·8	—	13·0
32	0·68	13·5	10·0	—	—
33	1·05	20·0	—	19·7	—
34	0·59	9·9	8·6	—	—

(*b*) *The effect of the anion on the relation between sodium influx and the external sodium concentration*

In the previous paper it was shown that there was a non-linear relation between the influx and the external sodium concentration. At an external concentration of about 1 mM./l. the influx approached a maximum rate. This behaviour could be due to rate-limitation imposed by the sodium-transporting system itself or it might be brought about by the resistance to anion movement determining the rate of sodium transport. These measurements were therefore repeated using sodium sulphate solutions in the place of sodium chloride. As is shown below there is no significant penetration of sulphate during the relatively short periods of these measurements. The values of the sodium influx at different external concentrations of sodium sulphate are shown in Fig. 2 for four different animals. The type of relationship is precisely the same as that found with sodium chloride solutions. Saturation is approached at 1 mM./l. and the half-saturation concentration (between 0·2 and 0·25 mM./l.) is similar to that found for chloride (Shaw, 1958, 1959). These results indicate that the behaviour of the sodium-transporting system is not influenced by the simultaneous penetration of the anion and that the limitation in the rate of transport is a property of the transporting system itself.

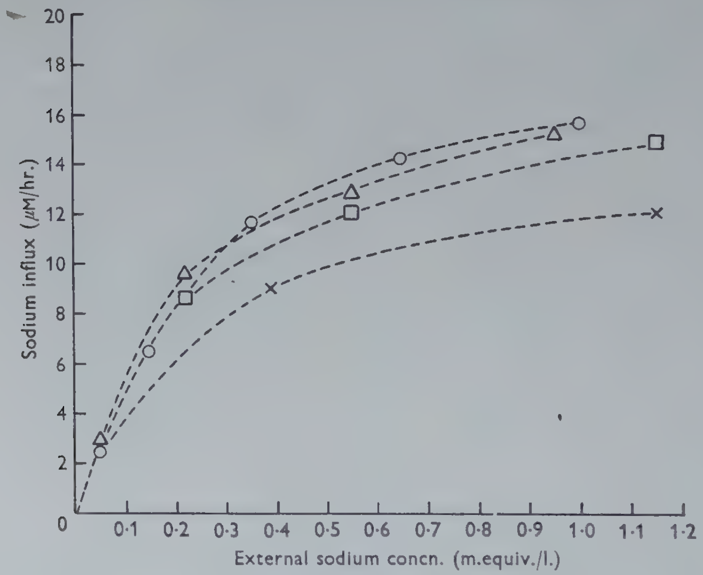

Fig. 2. The relation between the sodium influx and the external concentration of sodium sulphate.

(*c*) *The effect of the anion on the rate of net uptake of sodium*

With sodium chloride solutions, if the external sodium concentration exceeded the external equilibrium concentration, a net uptake of sodium took place. If the external concentration was not too high then the net uptake rate was approximately equal to the difference between the influx and the loss rate. At higher external concentrations the net uptake rate was lower than expected and this was probably due to the fact that an increasing proportion of the influx was involved in exchange diffusion (Shaw, 1959).

The effect of the nature of the anion on the extent of the net uptake of sodium was measured in four different animals and the results are shown in Table 2. Net

Table 2. *The effect of the nature of the anion on the net uptake of sodium from solutions of sodium salts*

Specimen no.	External sodium concn. (m.equiv./l.)	Net uptake of sodium (μM./hr.)	Anion
29	0·78	6·0	Sulphate
29	0·70	5·0	Nitrate
29	0·45	4·7	Sulphate
29	0·48	4·7	Chloride
29	0·48	4·5	Sulphate
32	0·72	6·8	Sulphate
32	0·70	3·1	Nitrate
32	0·50	6·2	Chloride
32	0·49	4·0	Sulphate
32	0·47	4·1	Sulphate
34	0·57	7·0	Chloride
34	0·61	5·2	Sulphate
34	0·60	6·7	Nitrate
35	0·62	4·8	Sulphate
35	0·70	5·5	Chloride
35	0·61	6·0	Nitrate

uptakes from solutions of sodium chloride, nitrate and sulphate were determined. For a given external concentration the net uptake rate is of the same general order from the different solutions. In two of the animals (specimens 32 and 34) the net uptake from sulphate was rather lower than from chloride. In the four animals it varied from 65 to 100% of the sodium chloride value. In the case of uptake from sodium sulphate, sodium is exchanged for other cations (see below) and it is possible that here exchange diffusion is quantitatively more important than is the case of uptake from sodium chloride. In any case exchange diffusion still accounts for only a small proportion of the total influx.

(*d*) *The penetration of sulphate during the net uptake of sodium from sodium sulphate solutions*

In order to investigate further the process in the absence of anion movement it was necessary to make use of a salt, the anion of which could be shown not to penetrate significantly in the course of the experiment. Sulphate appeared the most likely to fulfil this requirement and therefore the penetration of sulphate during a sodium-uptake experiment was measured. This was done in two ways. The first method involved measuring the movement of the anion using sulphate labelled with ^{35}S as an indicator. An animal was placed in a solution of the labelled sodium sulphate and the sodium concentration and the activity of the solution were recorded. The measurements were repeated at intervals over a period of about 6 hr. In some experiments potassium sulphate was used instead of sodium sulphate as a control. The results, shown in Table 3, demonstrate that in sodium sulphate, despite the net uptake of sodium, no decrease in the radioactivity of the solution occurred and, hence, there was no influx of sulphate.

The second method involved the measurement of the total strong acid anion concentration in the external solution by passing a sample through a strongly acidic

Table 3. *The uptake of* ^{35}S*-labelled sulphate from solutions of sodium and potassium sulphate*

Specimen no.	Salt	External Na concn. (m.equiv./l.)	Net uptake of sodium (μM./hr.)	Mean activity of ^{35}S in ext. solution (counts/min./ml.) Period 1 (0–2 hr.)	Period 2 (2–4 hr.)	Period 3 (4–6 hr.)
29	Na_2SO_4	0·48	4·5	6023	6321	6290
32	Na_2SO_4	0·468	4·1	6075	6298	6400
35	K_2SO_4	—	—	5921	6441	6354
36	Na_2SO_4	0·504	4·9	6211	6280	6447
37	K_2SO_4	—	—	6224	6224	6261

ion-exchange resin (Amberlite IR–120) and recording the conductivity of the effluent. This measured the conductance of strong acids derived from the anions in the solution (and eliminated bicarbonate). An animal was placed in a sodium sulphate solution and the anion concentration of the water was measured in this way. After some hours of sodium uptake the anion concentration was measured again. The results are shown in Table 4. It was found that the conductivity of the solution after passing through the exchange resin had slightly increased during the experimental period and this showed there had been no uptake of strong acid anions during the period—in fact, a slight loss had occurred.

Table 4. *Anion uptake from sodium sulphate*

(The effect of sodium uptake on the conductivity of the external solution after passing through an acidic exchange resin.)

Specimen no.	Initial sodium concn. (mM./l.)	Conductivity of external soln. (μmhos)	Initial conductivity of soln. through H-resin (μmhos)	Final sodium concn. (mM./l.)	Final conductivity of soln. through H-resin (μmhos)
64	0·52	33·5	117	0·393	119
65	0·497	33·5	117	0·313	122
67	0·509	32·5	115	0·375	121
68	0·485	31·5	110	0·381	113
69	0·474	31	110	0·370	112

From the experiments with labelled sulphate and from these experiments on the total anion concentration it can be concluded that sodium uptake from sodium sulphate solutions occurs in the complete absence of anion uptake, and, therefore, sodium transport involves an exchange with some other positively charged ion.

(*e*) *The rate of ammonia excretion during sodium uptake*

The rate of excretion of ammonia was measured before and during a period of sodium uptake. Salt-depleted animals were placed individually in 250 ml. of de-ionized water for a period of several hours. At the end of this time the ammonia concentration of the water was measured and this was taken to indicate the rate of ammonia excretion. The ammonia concentration of the water was still very low (about 0·1 mM./l.) and ammonia loss from the water was negligible as it was trapped in the water as bicarbonate. Sodium sulphate was now added to the water to bring the concentration to a level at which the animal would absorb sodium rapidly. Sodium uptake was allowed to proceed for another period of several hours and the ammonia concentration of the water measured again. In certain experiments potassium sulphate was used instead of the sodium salt to act as a control. The results are shown in Table 5. There was no increase in the rate of ammonia production during the period of sodium uptake—in fact, it was generally somewhat reduced. The same results were obtained in the controls with potassium sulphate. Hence sodium uptake in the crayfish is not accompanied by an additional excretion of ammonia.

Table 5. *The effect of the addition of sodium or potassium sulphate to the external solution on the rate of ammonia production*

Specimen no.	Rate of NH_3 production. 1st period (μM./hr.)	Salt added. (approx. 0·5 m.equiv./l.).	Net uptake of sodium (μM./hr.)	Rate of NH_3 production. 2nd period (μM./hr.)
29	2·2	Na_2SO_4	4·7	1·7
29	1·5	Na_2SO_4	4·5	1·0
29	1·3	K_2SO_4	—	1·6
32	3·9	Na_2SO_4	3·0	3·0
32	4·7	K_2SO_4	—	3·1
32	1·9	Na_2SO_4	4·1	0·8
36	5·3	Na_2SO_4	2·5	2·7
36	5·4	Na_2SO_4	3·7	2·9
36	3·1	K_2SO_4	—	2·8
37	3·6	Na_2SO_4	3·1	4·3
37	8·6	Na_2SO_4	4·9	3·1
37	3·7	K_2SO_4	—	2·6

(*f*) *The nature of the exchange mechanism*

Since it has been shown that the uptake of sodium from a sodium sulphate solution is not accompanied by the penetration of the anion, some other cation must be excreted into the external solution in equivalent amount. Therefore an analysis of this solution during sodium absorption should reveal the presence of the exchanging cation. The results given in the last section show that this cation is not represented in the solution by additional ammonium ion. The external solution was therefore analysed by two other methods with the object of revealing the nature of the exchange.

In the first method the ionic content of the external solution was measured by conductimetric analysis. It was found that when an animal was placed in a volume of de-ionized water the conductivity of the water gradually increased and this was due mainly to the accumulation of ammonium bicarbonate and, in smaller measure, other ions such as sodium and chloride. If a salt such as potassium sulphate (from which no ion uptake occurred) was added to the solution the conductivity jumped up and then continued to increase at about the same rate. If sodium sulphate was added instead, and a net uptake of sodium occurred, then the effect on the conductivity must depend on the nature of the ion exchange. Thus if the absorbed sodium was exchanged for some other metallic cation of similar mobility then the conductivity of the solution would continue to increase at the same rate: if the exchanged cation had a higher mobility then the conductivity would increase at a greater rate. In fact it was found that the increase in conductivity after the addition of sodium sulphate was always much slower than before. A typical result is shown in Fig. 3 where the effect of the addition of sodium sulphate on the rate of increase of conductivity is compared with the effect of potassium sulphate. In this particular case the conductivity increase completely ceased during sodium uptake. The results for seven different animals are shown in Table 6. In all cases the addition of sodium sulphate was followed by a drop in the rate of increase of conductivity which was not found with the potassium sulphate controls. These

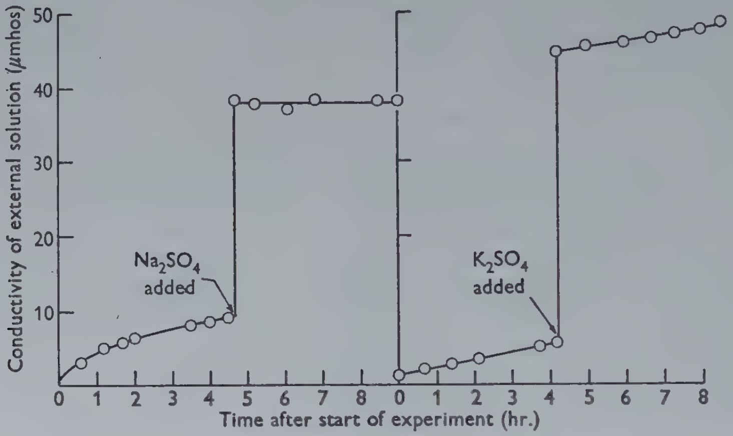

Fig. 3. The effect of the addition of sodium and potassium sulphates on the rate of increase of the conductivity of the external solution.

Table 6. *The effect of the addition of sodium or potassium sulphate to the external solution on the rate of increase of its conductivity*

Specimen no.	Rate of increase of conductivity. 1st period (μmhos/hr.)	Salt added	Net uptake of sodium (μM./hr.)	Rate of increase of conductivity. 2nd period (μmhos/hr.)
32	0·6	Na_2SO_4	3·0	0·3
32	0·9	K_2SO_4	—	0·8
34	2·0	Na_2SO_4	5·2	0·6
35	2·4	Na_2SO_4	4·8	0·5
35	0·8	K_2SO_4	—	0·8
36	1·5	Na_2SO_4	2·5	0·0
36	1·0	K_2SO_4	—	0·8
36	1·2	Na_2SO_4	3·7	0·1
36	0·8	K_2SO_4	—	0·8
37	0·8	Na_2SO_4	3·1	0·2
37	1·6	Na_2SO_4	4·9	0·2
37	0·6	K_2SO_4	—	0·6
63	0·9	Na_2SO_4	6·7	−0·4
64	0·7	Na_2SO_4	4·5	−0·3

experiments show that sodium uptake is not accompanied by the excretion of other metallic cations of equivalent mobility.

A similar conclusion follows from the second series of experiments. In these the external solution was analysed by measuring the conductivity of a sample which had passed through an ion-exchange column containing a strongly basic resin (Amberlite IRA–400). Since the resin replaces acid radicals by hydroxyl groups the measured conductivity of the sample is that of metallic hydroxides in solution and ammonia is eliminated. If the external solution contains sodium sulphate and samples are measured before and after a period of sodium uptake then the exchange of sodium for a metallic cation of similar mobility would be revealed by the fact that the conductivity of the two samples is the same. If the exchange is for some cation which is not retained in solution after passing through the resin (e.g. if it is not a metallic cation) then the conductivity of the second sample would be lower. When such measurements were made it was found that the conductivity of the second sample was always lower than the first. The interpretation of the actual values for the conductivity was complicated by the fact that some metallic cations (other than Na) were liberated into the external solution in the absence of sodium uptake. It had to be assumed that these cations were still excreted into the solution during sodium uptake and that they contributed to the final conductivity measurement. The actual experiments were threfore carried out as follows. The animals were first placed in a volume of de-ionized water and at the end of a known period the conductivity of a sample, passed through the resin, was measured. The sodium concentration was also measured and the conductivity of an equivalent solution of NaOH deducted from the conductivity of the sample. This value now represented the conductivity of the hydroxides of the metallic cations (other than Na) which were excreted by the animal during the period. The experiment was now repeated with the animals in a sodium sulphate solution. The conductivity of samples, passed through the resin, was measured before and after the period of sodium uptake. To the first of these measurements a correction was applied to take into account the normal metallic ion excretion during the period and the value of the correction was derived from the previous measurements on excretion into de-ionized water. The results of these experiments are shown in Table 7. In all cases sodium uptake was accompanied by a considerable fall in the conductance due to the metallic cations in the external solution and the decrease was consistent with the view that the absorbed sodium had not been replaced by the equivalent excretion of metallic cations.

These experiments therefore confirm the results of the previous ones and substantiate the conclusion that sodium uptake is not accompanied by an equivalent excretion of metallic cations.

The possibility remains that the bulk of the sodium is exchanged for cations which are normally excreted, without increasing their rate of excretion. Metallic cations can be eliminated at once as fulfilling an important role in such an exchange since their normal rate of excretion is much lower than the maximum rate of sodium uptake. It was found, however, that the normal rate of excretion of

Table 7. *The effect of the net uptake of sodium from sodium sulphate on the conductivity of the external solution after passing through Amberlite* IRA-400 *ion exchange resin*

(Conductivities in μmhos.)

Specimen no.	Conductivity of initial sample	Correction due to excreted metallic cations	Expected conductivity of final sample in absence in Na uptake	Conductivity of final sample	Net sodium uptake (mM.)	Expected conductivity of final sample if Na not exchanged for metallic cations
64	59·5	5·3	64·8	55	0·13	48·3
65	62	14·2	76·2	49	0·18	52·2
67	59·5	7·2	66·7	56	0·13	49·3
68	58	2·5	60·5	48	0·10	47·0
69	56	4·3	60·3	48	0·10	47·0

ammonium was of the same general order as that of sodium uptake and therefore ammonium ions might fulfil this role. It is easy to visualize an exchange taking place whereby ammonium, which is normally excreted together with bicarbonate, is exchanged for sodium during sodium uptake and bicarbonate is retained in the blood to balance the absorbed sodium. There is, however, at least one other possible explanation which equally well fits the facts, and that is that the sodium is exchanged for hydrogen ions. Now the presence of ammonium bicarbonate in the external solution would effectively buffer this solution against the small changes in hydrogen-ion concentration produced by such an exchange and thus changes in conductivity of the external solution due to this cause would not be detectable. Further, the behaviour of the external solution in the ion-exchange resin experiments could equally well be explained in these terms. It is by no means easy to distinguish between an exchange for ammonium ions or hydrogen ions if these continue to be produced at their normal rate. If an exchange for normally excreted ammonium ions is the correct explanation then it is obvious that the normal rate of ammonia excretion must always exceed, or at least equal, the rate of sodium uptake. These two rates are compared for a number of animals in Table 8. In most cases there was a general correspondence between sodium uptake and ammonia excretion but there were certain cases (specimens 29, 63 and 64) where the rate of sodium uptake definitely exceeded that of ammonia production. In these animals it is probable that a proportion, at least, of the sodium ions had exchanged for hydrogen ions, although it does not follow that this must normally be the case. It is just as likely than an exchange with ammonium ions is the normal mechanism but the animal can switch over to hydrogen-ion exchange when the ammonia excretion rate falls. Because of the intereactions between the three ions (i.e. NH_4^+, HCO_3^-, and H^+) it is not possible to distinguish between these two possible exchange mechanisms by an analysis of the external solution alone.

Table 8. *A comparison between the rate of ammonia production and the net uptake of sodium from sodium sulphate solutions*

Specimen no.	External sodium concn. (m.equiv./l.)	Net uptake of sodium (μM./hr.)	Mean ammonia production rate (μM./hr.)
29	0·45	4·7	1·9
29	0·50	4·5	1·2
29	0·48	4·5	0·6
32	0·49	3·0	3·2
32	0·47	4·1	1·3
34	0·61	5·2	7·0
35	0·62	4·8	8·0
36	0·48	2·5	3·8
36	0·53	3·7	4·1
36	0·50	4·9	4·5
37	0·48	3·1	3·7
37	0·52	4·9	5·8
63	0·56	6·7	3·2
64	0·53	4·5	2·0

DISCUSSION

It has been demonstrated that both the sodium influx at a given external concentration and the relation between the influx and the external concentration are not dependent on the presence of a penetrating anion. It is evident, therefore, that in the crayfish the process of sodium transport is basically one of ion exchange. It is shown in a later paper (Shaw, 1960) that the salt-depleted animal takes up sodium much more rapidly than chloride even when this ion is present. The mechanism of sodium transport is clearly quite independent of anion movement. In this respect the results obtained for the crayfish entirely confirm the earlier results of Krogh on other animals. The fact that sodium influx may differ markedly from anion influx is also in accord with the tracer studies of Jørgensen *et al.* (1954) on the frog. It is becoming evident that the behaviour of the isolated frog skin cannot be taken as a complete model on which to base our understanding of the transport of ions by whole animals.

The question of the nature of the ion exchange process naturally arises. The fact that ammonia excretion is not increased during sodium uptake appears to distinguish the crayfish from those animals studied originally by Krogh. However, in view of the preliminary nature of some of Krogh's observations it might be worth repeating these measurements under carefully controlled conditions. The results on the crayfish show conclusively that an exchange for metallic cations (such as, for example, potassium and calcium) play little or no part in the process and that the exchange must involve the normally excreted cations—i.e. ammonium and hydrogen ions—without increasing their rate of excretion.

The fact that the rate of ammonia production often roughly corresponds to the maximum rate of sodium uptake may point to a normal exchange between these two ions. However, the fact that, under certain circumstances, the sodium uptake may exceed ammonia production shows that exchange for ammonium ions cannot be obligatory although this does not rule out the possibility that this type of exchange may constitute the normal mechanism. There is no *a priori* reason for supposing that the exchange process must necessarily involve only a single ion species. It is not unlikely that both ammonium and hydrogen ions are excreted through the same channel and that sodium may be exchanged for either, depending on the relative rates of excretion and the type of metabolic activity displayed by the animal at the time.

SUMMARY

1. Sodium influx and net sodium uptake have been measured for the crayfish, *Astacus pallipes*, in solutions of a variety of sodium salts.

2. The nature of the anion has no effect on the sodium influx.

3. Sodium influx is related to the external sodium concentration in the same way whether the anion is chloride or sulphate.

4. The net sodium uptake rate from sodium sulphate is 65–100% of that from sodium chloride solutions.

5. In sodium sulphate solutions the sulphate ion does not penetrate.

6. There is no increase in ammonia excretion during net sodium uptake from sodium sulphate solutions.

7. An analysis of the external solution by conductivity and ion exchange showed that during sodium uptake sodium ions are not exchanged for metallic cations and must be exchanged either for ammonium or hydrogen ions without increasing their normal rate of excretion.

8. Sodium uptake may sometimes exceed ammonia production so that hydrogen-ion exchange may be involved in these cases.

9. The possibility is discussed that sodium ions exchange for either ammonium of hydrogen ions depending on the metabolic activity of the animal.

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THE ABSORPTION OF SODIUM IONS BY THE CRAYFISH *ASTACUS PALLIPES* LEREBOULLET

III. THE EFFECT OF OTHER CATIONS IN THE EXTERNAL SOLUTION

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INTRODUCTION

It is now well established that many aquatic animals can selectively absorb sodium ions from their environment. In sea water sodium is quantitatively the most important cation, but in natural fresh waters sodium ions may form only a small part of the total cation content. Calcium and magnesium ions, especially, may be present in concentrations considerably higher than that of sodium. It is important, therefore, to see what effect, if any, these other cations have on the rate of absorption of sodium by freshwater animals. In laboratory experiments on sodium uptake ammonium ions may also be present in relatively high concentrations since these are often excreted by aquatic animals and tend to accumulate in the limited volume of external solution used for the experiments. The effects of most of these cations on sodium absorption is not known. It is firmly established that sodium can be selectively absorbed from mixtures of sodium and potassium salts in the frog and goldfish (Krogh, 1939) and in cases where sodium influx has been measured by the tracer technique it has been found that the influx is not affected by the presence of potassium in the external solution (Koch & Evans, 1956, for *Eriocheir*; Ussing, 1954, for the frog skin; and Treherne, 1954, for *Aedes*).

The object of the work reported in this paper was to see to what extent sodium influx in the crayfish was influenced by the cation composition of the external solution.

MATERIAL AND METHODS

These were generally the same as described in previous papers (Shaw, 1959, 1960*a*). All the animals were previously salt-depleted by treating them with de-ionized water. To determine the effect of a given cation, the sodium influx from a sodium chloride solution of known concentration was first measured in the manner described previously (Shaw, 1960*a*). After a sufficient time had elapsed the cation in the form of solution of its chloride or sulphate was added to bring its concentration to the desired value. The experiment was then continued and the influx was measured again. The new influx (i.e. after the addition of the cation) was expressed as a percentage of the initial influx.

RESULTS

(*a*) *Potassium*

The effects of the addition of potassium salts on the sodium influx from 0·05 mM./l. NaCl solutions are shown in Fig. 1. 1 mM./l. concentrations of potasium chloride or sulphate had no effect in any of the experiments despite the fact that the concentration of potassium was twenty times that of sodium. In the higher potassium concentrations the results were less consistent but there was no general tendency for potassium to increase or decrease the influx. This behaviour is in accord with the effects of potassium on sodium influx found in other animals, and once again illustrates the selectivity of the sodium-transporting system.

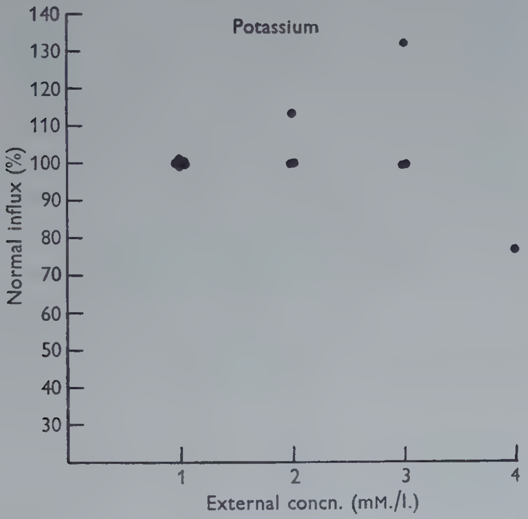

Fig. 1. The effect of potassium on the sodium influx.

(*b*) *Calcium*

The effect of calcium on sodium influx is shown in Fig. 2. At calcium concentrations lower than 1 mM./l. no effects were observed. At higher concentrations the influx was reduced to as low as 60% of the initial rate in certain cases, but this effect was not consistently observed. Over the range studied there were, in fact, more cases in which the influx was completely unaffected than in which it was reduced. There is no evidence that the reduction in influx is greater as the concentration increases and it is unlikely, therefore, that the occasional effects of calcium are due to the ions competing with sodium ions for transport. It may be that in high calcium concentrations the adsorption of calcium ions at the surface is sufficient in some cases to block some of the transporting sites. At the concentrations of calcium normally found in fresh water this ion is unlikely to affect sodium influx.

(c) *Magnesium*

The effect of magnesium ions on sodium influx is shown in Fig. 3. Again the results were rather inconsistent. In many cases magnesium was without effect but generally the sodium influx was definitely increased. In the most pronounced cases the new influx was practically three times the original rate, but, on the average, the increase was much smaller—amounting to about 30% of the initial rate. The inconsistency in the response often applied even to individual animals. Thus the influx in one animal might sometimes be affected by magnesium and yet at other times not affected at all. It was generally noted that an increase in the influx occurred if the initial influx was rather below normal and it is possible that the

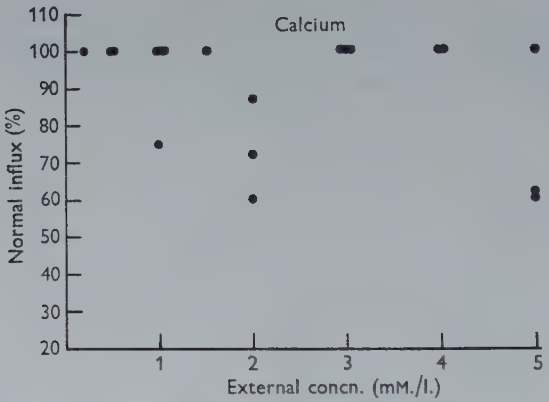

Fig. 2. The effect of calcium on the sodium influx.

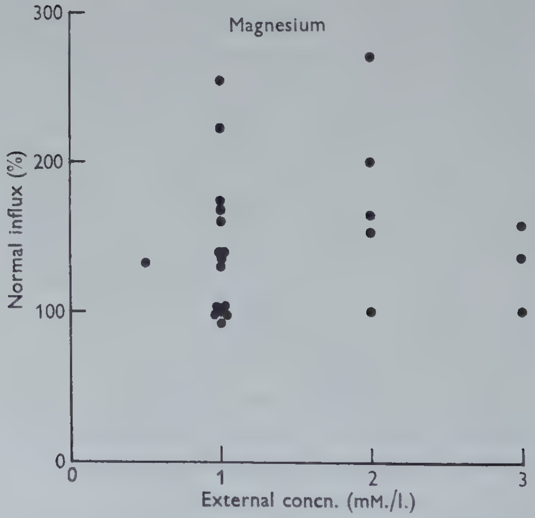

Fig. 3. The effect of magnesium on the sodium influx.

magnesium ions exert their effect by stimulating the activity of the transporting cells. The presence of magnesium in the external solution may, therefore, be important in maintaining the normal level of activity of the sodium-transporting system, although at the concentrations of magnesium normally found in fresh water this effect is not likely to be a very important one.

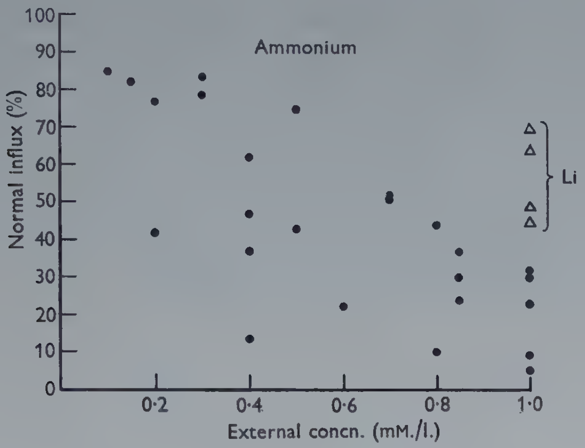

Fig. 4. The effect of ammonium ions on the sodium influx.

(*d*) *Ammonium*

The effect of free ammonium ions in the external solution on the sodium influx from 0·05 mM./l. sodium chloride is shown in Fig. 4. Compared with the effects of the other cations studied, the effect of ammonium ions was most marked. Ammonium ions produced a marked inhibition of sodium influx which at ammonium concentrations of 1 mM./l. amounted to a reduction of the influx to about 20% of its normal value. The effect of ammonium ions on the influx is clearly related to the external ammonium concentration, the influx decreasing with increasing concentration. It is interesting to note that ammonium ions were more effective than lithium ions at the same concentration in reducing the sodium influx. The effect of a 1 mM./l. lithium chloride solution is also shown in Fig. 4. At this concentration the sodium influx was reduced to between 50 and 70% of the initial value compared with a reduction to between 5 and 30% produced by ammonium ions. Lithium ions have been shown to reduce sodium influx in *Eriocheir* (Koch & Evans, 1956) and partially to replace sodium ions for transport in the frog skin (Zerahn, 1955) and there is little doubt that lithium can compete with sodium for transport. It may be argued that the same is true for ammonium ions in the crayfish, but before this idea can be considered it is necessary to rule out the possibility that the ammonia is simply exerting a toxic effect on the transporting cells. This seems unlikely for the following reasons:

(*a*) the inhibition is produced by very low concentrations of ammonia—lower in fact than that recorded for free ammonia in crayfish blood (Florkin & Frappez, 1940), (*b*) the same concentration of ammonium ions has no effect on the active transport of chloride ions (Shaw, 1960*b*), and (*c*) the inhibition can be abolished by simply increasing the external sodium concentration. This latter effect is illustrated in two animals in fig. 5. In these animals, with an external sodium concentration of 0·05 mM./l., 0·8 mM./l. ammonium chloride reduced the sodium influx to 20% and 40% of the normal respectively. As the external sodium concentration was increased the degree of inhibition became smaller until at a sodium concentration of 1 mM./l. the effect was almost negligible. It would thus appear that the ammonium ion must exceed the sodium ion concentration in the external solution by a ratio of 10:1 or more for the inhibition to be effective. This fact, together with the fact that ammonium ions exert their effect specifically on sodium influx (i.e. they are ineffective in the case of chloride influx) gives weight to the suggestion that ammonium ions are competing with sodium ions for available transport sites. If this is so then the transporting system can be characterized by its affinity for cations in the following order: sodium > ammonium > lithium.

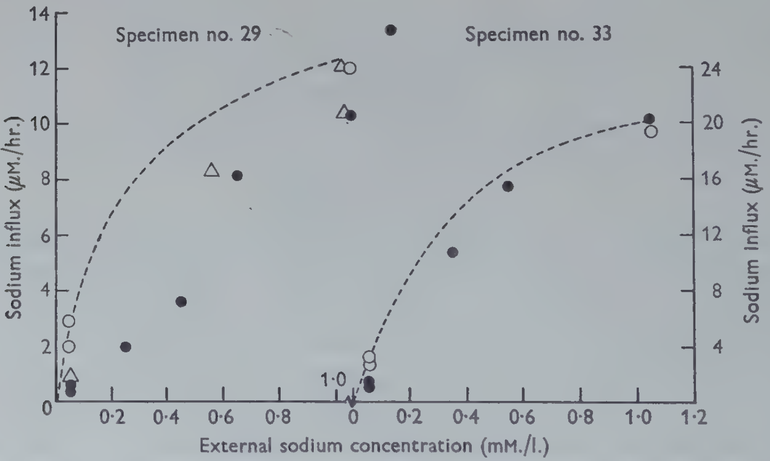

Fig. 5. The effect of the external sodium concentration on the inhibition of sodium influx produced by ammonium ions. Open circles represent the normal sodium influx: closed circles, the sodium influx in the presence of 0·08 mM./l. ammonium chloride: and triangles, the sodium influx in the presence of 0·05 mM./l. ammonium chloride. The dotted lines represent the expected relation between the normal sodium influx and the external sodium concentration.

Table 1. *The effect of ammonium ions on the rate of sodium loss and the equilibrium concentration*

	Normal		In the presence of 1 mM./l. NH_4Cl				
Specimen No.	Eq. concn. (mM./l.)	Loss rate (μM./hr.)	New eq. concn. (mM./l.)	Loss rate (μM./hr.)	Influx from 0·17 mM./l. NaCl (μM./hr.)	Net uptake from 0·17 mM./l. NaCl (μM./hr.)	Loss rate by difference (μM./hr.)
29	0·015	0·76	0·058	0·63	1·75	1·05	0·70
32	0·021	0·77	0·067	0·76	2·05	1·24	0·81

Whether ammonium ions are actually transported inwards or not cannot be decided from these experiments—it is possible that they simply interfere with the normal attachment of sodium ions to the transporting system.

Ammonium ions only affect the sodium influx. They have no effect on the rate of sodium loss even when the influx is reduced to a low level. This is illustrated in Table 1 for two animals. The loss rate was calculated either by equating it with the influx at the external equilibrium concentration or by the difference between the influx and the rate of net uptake of sodium when this took place. Although the presence of ammonium ions had reduced the influx to 20–30% of its normal value, there was no significant effect on the rate of sodium loss.

Table 2. *The effect of some substituted ammonium chlorides on sodium influx*

Specimen no.	Methyl ammonium chloride	Dimethyl ammonium chloride	Ethyl ammonium chloride	Tetraethyl ammonium chloride	Choline chloride
29	100	150	—	140	200
32	100, 100	100	—	140	150
34	—	—	—	100	151
35	100	—	100	131	100
36	83	100	—	—	
37	61, 100	100	100	—	—

(*e*) *Substituted ammonium ions*

Sodium and ammonium ions resemble each other quite closely in electronic structure but the ammonium ion is complicated by the presence of the three protons from the hydrogen atoms. It is of interest to see the effect of compounds closely related to ammonia and differing only in the fact that one or more of the protons are replaced by larger nuclei. The compounds used ranged from the simplest substituted ammonia, methyl ammonium chloride, to the completely substituted compounds, tetra-ethyl ammonium chloride and choline chloride. The results of these experiments are shown in Table 2 where the sodium influx from a 0·05 mM./l. sodium chloride solution in the presence of 1 mM of the substituted ammonium compound is shown as a percentage of the normal influx. No reduction in influx similar to that produced by ammonium ions was observed with any of the substituted compounds. In two out of five of the experiments with methyl ammonium chloride a slight reduction of the influx was found, although in the case where this inhibition was greatest (specimen no. 37) it was not found again when the experiment was repeated. No inhibition occurred with any of the other substituted compounds. With the fully substituted compounds the influx was generally slightly increased—an effect rather reminiscent of that produced by magnesium ions.

(*f*) *Hydrogen ions*

The action of hydrogen ions on a specific sodium-transporting system constitutes rather a special case since they may be expected to affect the system in more than one way. If they are present in high concentration they may combine with the system and displace sodium ions; on the other hand, they may affect the configuration of the system in such a way that it no longer retains its specific property of combining with sodium ions. In the latter case the effect would be analogous to that of pH on the combining properties of an enzyme.

The effects of the pH of the external solution on the sodium influx are shown in Fig. 6. The pH has no effect in the range of pH 6–10 but below 6 the influx falls off sharply and at a pH 4 it is reduced to 20–30% of the normal value. pH 4 corresponds to a concentration of free hydrogen ions of 0·1 mg. ions/l. and may be compared with the concentration of 1·0 mg. ions/l. required for ammonium ions to produce a similar degree of inhibition.

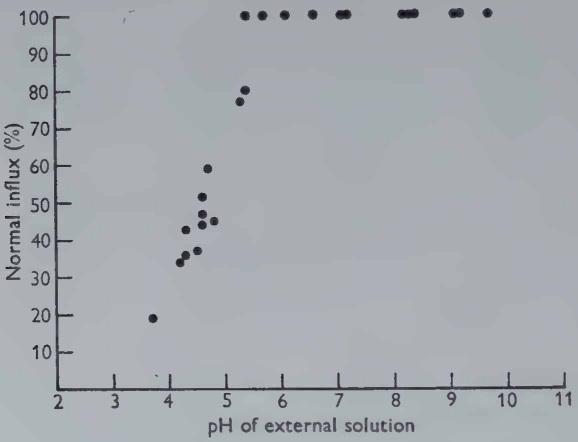

Fig. 6. The effect of external pH on the sodium influx.

Table 3. *The effect of hydrogen ions in the external solution on the rate of sodium loss*

	Normal			At pH 4·5		
Specimen no.	Influx (μM./hr.)	Net uptake (μM./hr.)	Loss rate (μM./hr.)	Influx (μM./hr.)	Net uptake (μM./hr.)	Loss rate (μM.(1 hr.)
29	1·24	0·8	0·44	0·55	0·0	0·55
32	1·27	0·72	0·55	0·46	0·0	0·46
39	2·66	−0·73	3·39	1·84	−1·92	3·76
40	1·53	0·45	1·08	0·83	−1·60	2·43

The effect of pH on the sodium influx is similar to that found for the isolated frog skin (Ussing, 1949; Schoffeniels, 1955). Ussing interpreted the effect of pH on the outside of the frog skin as being due to alterations in the permeability of the outside surface layer and suggested that this was a pace-setting factor for sodium influx. However, measurements of sodium outflux at low pH in the outside solution by Schoffeniels (1955) show that at pH 4 and above the outflux is little changed and at a lower pH it is greatly increased. Measurements of the loss rate in the crayfish, shown in Table 3, pointed to the fact that at pH 4 the loss was not decreased and, hence, presumably the surface permeability was also not decreased. These results are consistent with the view that the external pH directly affects the sodium-transporting system, but it is not possible in this way to distinguish between the effects of pH in interfering with sodium uptake directly, in changing the combining properties of the transporting system, or in depressing the general metabolism of the transporting cells.

DISCUSSION

It has been shown that sodium uptake in the crayfish is basically a process of ion exchange (Shaw, 1960*a*), but the effects of cations on sodium influx show that the process is quite dissimilar to that, for example, found for a typical ion-exchange resin. In this case cations are generally taken up according to the lyotropic series; the sodium-transporting system has little affinity for calcium and magnesium, and further, readily distinguishes between sodium and potassium. The specific nature of the sodium-transporting system is clearly demonstrated. The effects of hydrogen ions on the sodium influx are not unexpected, but the marked inhibition produced by ammonium ions is surprising. Evidence has been presented that the ammonium ions compete with sodium ions and it is curious that the obviously highly selective sodium transport system should be inhibited by the one common cation which is excreted in quantity by the animal itself. It is possible, of course, that this is purely a coincidence, but it is strange that the evolution of the highly selective system should stop short at extending selectivity to the ammonium ion. Since it has been shown that during sodium uptake (in the absence of a penetrating anion) the sodium ions are exchanged for ammonium or hydrogen ions which are normally excreted, it may be thought more probable that the effect of ammonium ions on the sodium influx is associated with this basic exchange mechanism. It would not be unexpected that if sodium ions were exchanging for ammonium ions, high concentrations of the exchanging ion in the outside solution would inhibit the exchange. Thus, for example, if sodium transport is mediated by a carrier, as is popularly supposed, then a net transfer of sodium would take place if the affinity of the carrier for sodium and ammonium ions was different at the outside and inside surfaces of the transporting membrane. At the inside the carrier affinity would be high for ammonium and low for sodium so that sodium was released and exchanged for ammonium. At the outside the reverse situation would be found; the affinity for sodium would be high and this ion would be taken up and displace ammonium. The presence of a high concentration of ammonium on the outside would prevent the ammonium ions on the carrier being displaced by sodium ions in the outside solution.

SUMMARY

1. The effect of various cations in the external solution on the sodium influx in the crayfish, *Astacus pallipes*, has been studied.

2. Potassium in concentrations up to 4 mM./l. has no significant effect on the sodium influx from 0·05 mM./l. NaCl solutions.

3. Calcium has no effect on the influx in concentrations up to 1 mM./l. At higher concentrations the influx may be reduced in some cases.

4. Magnesium generally increases the influx by about 30%. The effect is not related to the external concentration.

5. Ammonium ions reduce sodium influx. With an ammonium/sodium concentration ratio of 20:1 the influx is reduced to about 20% of normal. Ammonium ions do not affect the sodium loss rate.

6. Simple substituted ammonium compounds have little effect on the influx.

7. The external hydrogen ion concentration reduces sodium influx if the pH is below 6. At pH 4 the influx is reduced to 20–30% of normal. A low pH does not decrease the rate of sodium loss.

8. The nature of the specific inhibition of sodium influx by ammonium ions is discussed. It is suggested that the ammonium ions interfere with a normal sodium-ammonium exchange mechanism.

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THE ABSORPTION OF CHLORIDE IONS BY THE CRAYFISH, *ASTACUS PALLIPES* LEREBOULLET

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INTRODUCTION

Although sodium uptake has now been demonstrated in a number of freshwater animals and the active nature of the process shown conclusively in the isolated frog skin (Ussing, 1949*a*; Ussing & Zerahn, 1951) and in the intact animal (Jørgensen, Levi & Zerahn, 1954), the situation with regard to the uptake of chloride is not so clear. The classical studies of Krogh (1937*a*, *b*, 1938, 1939) showed that in a number of salt-depleted animals (for example, in *Eriocheir sinensis*, *Carassius auratus* and *Rana esculenta*) chloride was absorbed not only from solutions of sodium chloride but also from solutions of potassium, ammonium and calcium chlorides. In the last three solutions chloride was not absorbed so rapidly as from sodium chloride nor was the concentration reduced to such a low level. Krogh emphasized the independent nature of the mechanism for the uptake of sodium and chloride. In the isolated frog skin, on the other hand, Koefed-Johnsen, Levi & Ussing (1952) showed that chloride movement was passive and dependent on the simultaneous movement of sodium ions, although later Jørgensen *et al.* (1954) showed that in the intact animal active chloride transport could take place. Thus in the salt-depleted frog a net uptake of chloride from a potassium chloride solution was found despite the fact that the potential across the skin was lower than the equilibrium potential for chloride. The net chloride movement was thus in a direction opposite to that expected on the grounds of a purely passive movement of chloride.

The investigations reported in this paper are concerned with the nature of the factors which determine the uptake of chloride in the crayfish, *Astacus pallipes*.

MATERIAL AND METHODS

With the exception of those described below, the methods were the same as described in previous papers (Shaw, 1959, 1960*a*).

The chloride concentration of the external solution was determined by electrometric titration in a manner similar to that described by Sanderson (1952). Use of a micrometer burette enabled the chloride concentration in 3 ml. samples to be determined down to a level of about 0·005 mM./l. If the concentration was lower than this it was recorded as zero.

Measurements of potential difference were made by means of two calomel electrodes. One electrode made direct contact with the external solution: the other made contact with the dorsal blood sinus by means of a bridge consisting of a narrow polythene tube filled with sodium chloride at the same concentration as the blood. The polythene bridge terminated in a glass tip which was inserted into the blood sinus through a small hole in the dorsal carapace.

The chloride influx was measured by means of the isotope ^{36}Cl in the apparatus previously described (Shaw, 1960*a*). The Cl isotope, supplied in the form of HCl, was neutralized with either NaOH or KOH and was added to the circulating fluid together with sufficient non-radioactive salt to give the desired chloride concentration. Owing to the low specific activity of the chloride isotope and the low external concentrations used, the chloride influx could not be calculated in the manner described previously (Shaw, 1959). This was because of the small number of counts recorded during a short period of counting. If the counting period was extended then an appreciable uptake of chloride occurred during the actual count. The influx was therefore measured by counting during the whole period of the experiment and calculating the influx from an integrated form of the flux equation used previously. Thus if y_0 is the initial activity of the solution (in counts/min.), M is the influx and A the total amount of chloride in the solution, then at time t the new activity (y) is given by

$$y = y_0 \exp\left(-\frac{M}{A}t\right).$$

Now let N_1 be the total count recorded after time T_1. Then

$$N_1 = y_0 \int_0^{T_1} \exp\left(-\frac{M}{A}t\right) dt = y_0 \frac{A}{M}\left[1-\exp\left(-\frac{M}{A}T_1\right)\right]. \qquad (1)$$

Now y_0 likewise cannot be measured accurately in a short time, so let the total count (N_2) be recorded for a further period of time and the total time of recording now be T_2.

Hence $$N_2 = y_0 \frac{A}{M}\left[1-\exp\left(-\frac{M}{A}T_2\right)\right]. \qquad (2)$$

Equations (1) and (2) can now be solved for M.

Dividing (1) by (2), $$\frac{N_1}{N_2} = \frac{1-\exp\left(-\frac{M}{A}T_1\right)}{1-\exp\left(-\frac{M}{A}T_2\right)}.$$

Hence $$\frac{N_1}{N_2}\left[1-\exp\left(-\frac{M}{A}T_2\right)\right] = 1-\exp\left(-\frac{M}{A}T_1\right). \qquad (3)$$

Now equate each side to x. Then

$$\ln\left(1-\frac{N_2}{N_1}x\right) = -\frac{M}{A}T_2 \qquad (3a)$$

and $$\ln(1-x) = -\frac{M}{A}T_1, \qquad (3b)$$

hence
$$\frac{\ln(1-N_2/N_1\,x)}{\ln(1-x)} = \frac{T_2}{T_1}. \tag{4}$$

Now various solutions can be found for equation (4) depending on the value of T_2/T_1. The simplest solution is if $T_2/T_1 = 2$ and for simplicity of calculation it was generally convenient to arrange the experiment so.

Then if $T_2/T_1 = 2$,
$$\ln\left(1-\frac{N_2}{N_1}x\right) = 2\ln(1-x),$$

and
$$1-\frac{N_2}{N_1}x = 1-2x+x^2,$$

hence
$$x = 2-\frac{N_2}{N_1}.$$

Substituting in equation (3*b*)
$$\ln\left(1-2+\frac{N_2}{N_1}\right) = -\frac{M}{A}T_1.$$

Hence
$$M = \frac{A}{T_1}\ln\left(\frac{N_1}{N_2-N_1}\right). \tag{5}$$

For an influx measurement, the animal was allowed to settle down in the solution and then the counting was started. After a time of 1 hr. or, sometimes, 2 hr. (T_1), the total count was recorded (N_1). The experiment then continued for another period of equal length (total time T_2) and the total count recorded again (N_2). The influx (M) was then calculated from equation (5).

RESULTS

(*a*) *Rate of loss of chloride*

The rate of loss of chloride ions from the animal was measured by the rate of increase of the chloride concentration of the external medium. Individual animals were placed in 250 ml. of de-ionized water and the chloride concentration of this fluid was measured over the first few hours, i.e. before the concentration approached the equilibrium value. At the same time the sodium concentration was also measured so that it was possible to compare the rate of loss of the two ions. The results are shown in Table 1. The mean sodium loss rate into de-ionized water (1·2 μM./hr./10 g. body weight) for these animals is comparable, though slightly lower, than reported previously (Shaw, 1959). The rate of chloride loss in all the animals was somewhat below that of sodium although the general order of permeability was the same. The mean chloride loss rate is about 72% of that of sodium and the difference is just significant ($t = 2{\cdot}2$, $P = 5\%$).

(*b*) *Equilibrium concentration*

At a particular blood concentration and at a certain external concentration the animal achieves salt balance. In the salt-depleted animal there is a minimum value of the external concentration at which balance can be achieved (Shaw, 1959). In

Table 1. *Rate loss of chloride in de-ionized water*

Specimen no.	Weight (g.)	Rate of loss of sodium (μM./hr.)	Rate of loss of chloride (μM./hr.)	Specific Na loss rate (μM./hr./10g. body wt.)	Specific Cl loss rate (μM./hr./10g. body wt.)
48	10·1	1·13	0·68	1·12	0·67
49	10·2	0·95	0·60	0·93	0·58
50	10·5	1·09	0·9	1·04	0·86
51	15·3	2·36	1·45	1·54	0·95
52	9·0	—	0·7	—	0·78
54	16·1	—	1·1	—	0·68
55	17·3	—	1·19	—	0·69
56	8·5	—	1·25	—	1·46
			Mean	1·16	0·83

Table 2. *External equilibrium concentration for salt-depleted animals*

Specimen no.	External sodium concn. (mM./l.)	External chloride concn. (mM./l.)
40	0·052	0·023
41	0·057	0·009
42	0·086	0·048
44	0·086	0·048
45	0·060	0·018
46	0·040	0·027
48	0·073	0·033
49	0·041	0·037
50	0·052	0·005
Mean	0·061	0·028

the case of sodium ions this was found to be 0·04 mM./l. The same must also be true for chloride ions—i.e. there must be a situation where the loss is just balanced by an equal uptake and the external concentration at which this occurs is not necessarily the same as for the sodium ion. The minimum external concentrations for balance for sodium and chloride ions were compared in a number of animals and are shown in Table 2. The mean value for sodium is comparable with that found previously and is significantly higher than that found for chloride ($t = 4{\cdot}2$, $P < 1\%$). This might be explained by the lower permeability to chloride, although in certain cases (for example animals 41 and 50) the minimum equilibrium concentration is so low that some other explanation is probably required here.

(*c*) *Net uptake of chloride*

It has been shown that when a salt-depleted animal is placed in a solution containing a concentration of sodium higher than the equilibrium concentration then a net uptake of sodium takes place (Shaw, 1959). In similar experiments the net uptake of chloride was measured from a solution of approximately 0·3 mM./l. NaCl and was compared with the simultaneous net uptake of sodium. The net uptake of the two ions was measured from the rate of fall of the external concentration. The results of these experiments are shown in Fig. 1 and they show two rather surprising features. First, the rate of net uptake of chloride is invariably considerably below the rate of net uptake of sodium. Thus during the first day of the experiments the mean rate of sodium uptake is 1·7 μM./hr. (range, 1·3–2·0 μM./hr.) compared with the mean rate of chloride uptake of only 0·5 μM./hr. (range, 0·2–1·0 μM./hr.). It is unlikely that the difference in the permeability of the body surface to the two ions alone could account for this fact since the ratio of net uptake of sodium to chloride is 3·5 compared with the permeability ratio (calculated from the respective loss rates) of only 1·4. Secondly, in some cases a net uptake of chloride continued after the net uptake of sodium had ceased and in at least one case the uptake of chloride continued during a period of definite sodium loss.

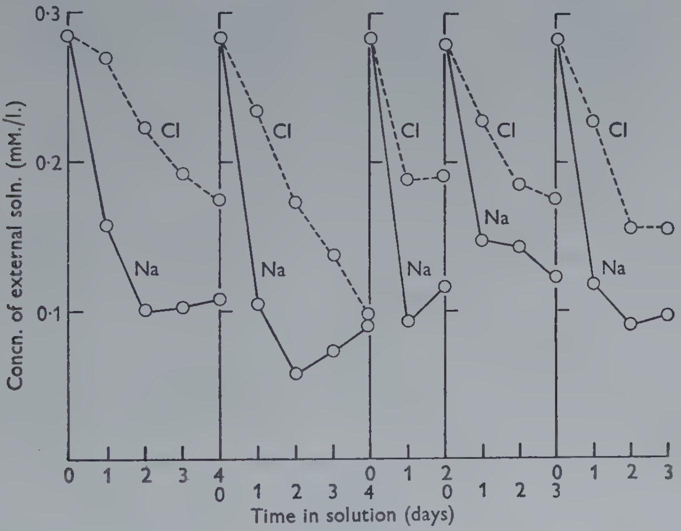

Fig. 1. The net uptake of sodium and chloride from sodium chloride solutions by five salt-depleted animals.

These experiments emphasize once more the independence of the sodium uptake mechanism (Shaw, 1960*a*) even in the presence of an equal concentration of chloride ions. Clearly the rate of sodium uptake is not limited by the rate of simultaneous penetration of chloride. The experiments do not, however, answer the question as to the extent to which chloride uptake depends on simultaneous sodium penetration. For this it is necessary to study the net uptake of chloride from solutions from which sodium is absent. For this purpose solutions of potassium or calcium chloride were used, the experiments being carried out as before, each animal being placed in 250 ml. of an approximately 0·3 mM./l. and the chloride concentration of the solution being measured over a period of a few days. The results obtained with eight different animals in KCl solutions are shown in Fig. 2. Essentially similar results were obtained with calcium chloride solutions. Only in a small number of cases was a net uptake of chloride observed at all and, even then, it was at a considerably slower rate than from the sodium chloride solutions of the same concentration. In some cases, a considerable net loss of chloride was observed. If an independent mechanism for chloride uptake was present it was clearly working at a very low level and generally only just sufficient to balance the normal chloride loss.

The effect of the presence of sodium ions in the external solution in increasing the rate of net uptake of chloride is further illustrated by the effect of the addition of sodium sulphate on the net uptake of chloride from potassium chloride solutions. This is shown, for example, for two animals in Table 3. The addition of sodium greatly increased the net uptake rate, the new rate exceeding the old by a factor of ten or more.

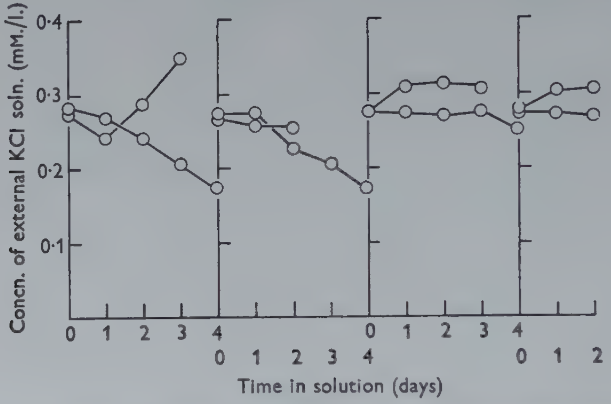

Fig. 2. The net uptake of chloride from potassium chloride solutions by salt-depleted animals. Points are joined where they represent measurements on the same animal.

Table 3. *Effect of addition of sodium sulphate to the external solution on the net uptake of chloride*

Specimen no.	External soln.	Concn. (mM/l.)	Net uptake rate 1st day (μM./hr.)	Net uptake rate 2nd day (μM./hr.)	Concn. of Na_2SO_4 added (m.equiv./l.)	Net uptake rate 1st day (μM./hr.)	Net uptake rate 2nd day (μM./hr.)
45	KCl	0·3	0·1	0·05	0·67	1·2	—
48	KCl	0·3	0·02	0·05	0·3	0·7	1·0
49	NaCl	0·08	0·2	0·05	0·2	0·5	—

The salt-depleted crayfish, then, can take up chloride from an external solution containing both sodium and chloride ions as long as these exceed the equilibrium concentration, but in the absence of sodium, the chloride net uptake is very slow or non-existent.

In this respect the behaviour of the crayfish appears to be different from that found by Krogh for a number of other freshwater animals. He recorded a net uptake of chloride from a number of different chloride salts, although the rate of uptake was generally not as rapid from sodium chloride (Krogh, 1939).

(*d*) *Chloride influx*

The chloride influx from sodium chloride solutions, measured with the isotope ^{36}Cl and related to the external concentration of chloride, is shown in Fig. 3. The low specific activity of the chloride isotope prevented accurate measurements of influx being made at this very low external concentration at which chloride balance could be maintained. The very high values for chloride influx are striking. It would appear, for example, that at the minimum equilibrium concentration the influx is higher than would be expected from the measurements of the loss rate. Also at an external concentration of 0·3 mM./l. the influx is much higher than expected from the net uptake rate at this concentration (see previous section). The influx at this concentration is between 7 and 10 μM./l., whereas the net uptake rate was only 0·5 μM./hr. and this difference is much greater than can be accounted for by the measured rate of chloride loss. The relation between the influx and the external concentration appears to be a similar kind to that found for sodium (Shaw, 1959). The relation is a non-linear one and probably reaches a maximum value at higher concentrations.

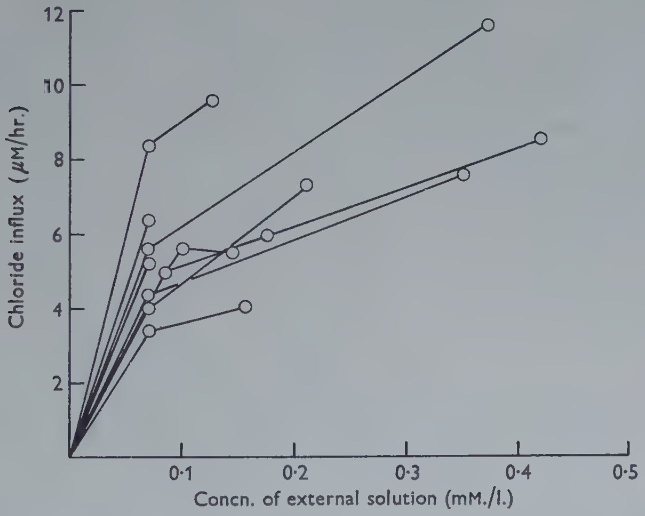

Fig. 3. The chloride influx from sodium chloride solutions. Points are joined where they represent measurements on the same animal.

Table 4. *The effect of a high sodium concentration on the chloride influx*

External soln.	Concn. (mM./l.)	Cl influx (μM./hr.)	Sodium salt added	Concn. (m.equiv./l.)	New Cl. influx (μM./hr.)
KCl	0·14	6·0	Na_2SO_4	1·0	9·8
NaCl	0·14	6·0	$NaHCO_3$	1·0	9·6

In the previous section it was shown that the presence of sodium in the external solution greatly increased the net uptake of chloride; a similar effect would be expected on the chloride influx. Such an effect is shown in Table 4 where the addition of 1 m. equiv./l. of Na is seen to increase the Cl influx from 0·14 mM./l. solutions of both NaCl and KCl by about 60%. It should, however, be noted that the addition of a similar concentration of sodium increased the net uptake by over ten times.

The fact that a high sodium concentration may increase the chloride influx suggests that part, at least, of the influx is due to passive chloride movement following sodium transport. Indeed all the results presented so far can be explained on the assumption that chloride enters along with sodium and that an independent chloride-transporting system, if present, only operates at a very low level. If this interpretation is correct then one would expect the chloride influx from potassium chloride solutions to be much lower than was found for sodium chloride solutions but this, in fact, was not the case. Measurements of chloride influx from potassium chloride solutions in relation to the external chloride concentration are shown in Fig. 4. It will be seen that the influxes from concentrations up to about 0·2 mM./l. are not low but are of precisely the same order as was found for sodium chloride solutions. It appears that at higher external concentrations the chloride influx from potassium chloride is lower than from sodium chloride and this would accord with the effects of high sodium concentrations on the Cl influx noted above. The fact that the chloride influx from potassium chloride solutions is high must be taken as evidence of independent chloride movement and it is difficult to explain this without invoking an independent chloride-transporting system. The proof that such a system exists is given below. It may be noted here that although the relation between chloride influx and external chloride concentration has the same general form as for the sodium system there are some important differences which point to special chracteristics of a specific chloride system. Thus the maximum influx is reached at a lower concentration than in the sodium system and the half-saturation concentration (which appears to be lower than 0·1 mM./l. for chloride—see Fig. 4) is also lower than the 0·2–0·3 mM./l. which is characteristic of sodium (Shaw, 1958, 1959, 1960).

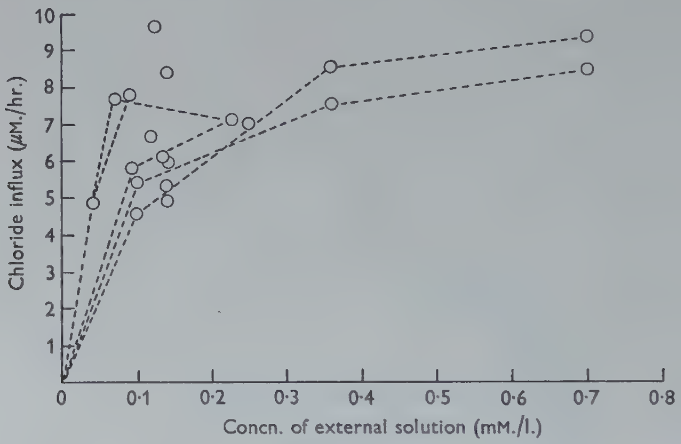

Fig. 4. The chloride influx from potassium chloride solutions.) Points are joined where they represent measurements on the same animal.

Comparing now the chloride influx from KCl (Fig. 4) and the net uptake of chloride (Fig. 2) from the same solution, we see that very little of the inwardly transported chloride can be retained. Now, since the rate of loss of chloride in the absence of chloride influx is low it must follow that as the external chloride concentration is increased so the rate of loss is also increased. This suggests that a large part of the chloride loss under these conditions is dependent on chloride influx and thus must be accounted for by exchange diffusion or a similar influx-dependent exchange. This exchange must account for as much as 90% of the chloride influx.

The function of a transporting system where a large proportion of the flux is involved in exchange for the same ion as it transports requires some comment. Although at first sight this transport seems useless, it is possible that such a system represents a potentially active transporting system, which is inactive under the particular conditions. Here the inactivation results not in a depression of the general flux level but in a switch from useful transport of chloride to chloride-chloride exchange. A hint that this may be the case in the chloride system of the crayfish comes from the fact that high sodium concentrations may greatly increase the net chloride uptake without increasing the chloride influx to the same extent (Tables 3 and 4). It is possible that the effect of the high sodium concentration is not simply to drag through additional chloride directly, but to make good the blood sodium deficit in the animal and, hence, to create a situation where the animal is deficient in chloride only. This situation may then provide the right conditions for the activation of the chloride-transporting system. The essential correctness of this view is shown by the results in the following section.

(*e*) *Chloride uptake by animals deficient in chloride only*

To obtain the conditions where the animals were deficient in chloride only, they were first washed out in de-ionized water in the usual way. They were then placed in a solution of sodium sulphate (concn. about 0·5 m.equiv./l.) for several days, the solution being replaced periodically. During this time the animals each reabsorbed something in the order of 100 μM. of sodium and hence regained the sodium which had been lost during the initial washing-out period. Since chloride had also been washed out, but not regained, the animals were now deficient in chloride. The net uptake of chloride in these animals from potassium chloride solutions was followed over a period of 2 days, and at the same time measurements of the uptake or loss of sodium and potassium were also made. The results of these experiments are recorded in Table 5. The effect of this treatment on the net chloride uptake was most striking. From an external solution of about 0·1 mM./l. potassium chloride, the net uptake rate during the first 8 hr. was between 1·5 and 3·1 μM./hr. and in 22 hr. the external concentration had been reduced virtually to zero. The net uptake was much faster than previously recorded from potassium chloride by the salt-depleted animals and, in fact, was faster also than from the sodium chloride solutions (cf. Fig. 1). During this rapid net uptake of chloride there was actually a net loss of sodium and in all but one case (specimen 46) there

Table 5. *The net uptake of chloride from potassium chloride solutions by chloride-deficient animals*

Specimen no.	Initial K concn. (mM./l.)	Initial Cl uptake after 2 hr. (μM.)	Net Cl uptake after 8 hr. (μM.)	External Cl. concn. after 22 hr. (mM./l.)	Net K uptake after 22 hr. (μM.)	Net Na loss after 22 hr. (μM.)
45	0·10	—	12·5	0·00	−0·5	11·7
46	0·099	—	19·2	0·00	+9·0	8·0
48	0·104	4·0	12·5	0·018	−1·7	12·0
49	0·102	6·7	17·5	0·00	0·0	10·0
50	0·090	6·7	21·5	0·00	−1·0	10·0
54	0·096	8·5	25	0·00	−1·5	18·0

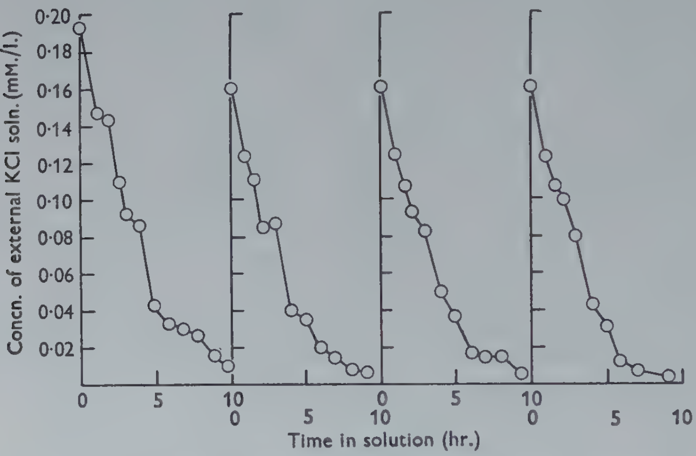

Fig. 5. The net uptake of chloride from potassium chloride solutions by four chloride-deficient animals.

was no net uptake of potassium—even in this case the net uptake of chloride greatly exceeded that of potassium. The chloride-transporting system had clearly been activated by this procedure and the chloride uptake was quite independent of the presence of sodium or of the penetration of other cations. A special feature of the activated chloride mechanism was its effectiveness at very low external chloride concentrations. This was clearly demonstrated by experiments where the chloride concentration of the external solution was measured at regular intervals during the net uptake of chloride from potassium chloride solutions. In this way the rate of net uptake of chloride could be related to external chloride concentration while this was being steadily reduced to zero. The results for four individuals are shown in Fig. 5, where the decrease of the external chloride concentration with time is shown. The slope of the curve at any given external concentration is a measure of the rate of net uptake of chloride at that concentration. It can be seen that the rate of net uptake at the higher concentration is maintained down to concentrations as low as 0·04 mM./l. or even down to 0·01 mM./l. After this the rate of uptake declines but continues until the external concentration is effectively reduced to zero.

It may be noted that in these experiments the rate of net chloride uptake was in the order of 6 μM./hr.—rather higher than in the previous experiments—and the value approaches those for the chloride influx from KCl as shown in Fig. 4. It seems to be a common property both of the influx and the net uptake that high rates are achieved at very low external concentrations. This suggests that the chloride-transporting system has a very high affinity for chloride ions and clearly distinguishes this system from that transporting sodium.

Table 6. *The effect of the external chloride concentration on the influx and net uptake of chloride from a potassium chloride solution*

KCl concn. (mM./l.)	Cl influx (μM./hr.)	Cl net uptake rate (μM./hr.)
0·094	7·0	5·0
0·298	7·0	4·0

Table 7. *A comparison between the chloride influxes from* KCl *solutions in animals deficient in sodium chloride and deficient in chloride only*

Specimen no.	KCl concn. (mM./l.)	Cl influx. NaCl-deficient animal (μM./hr.)	Cl influx. Cl-deficient animal (μM./hr.)	Cl net uptake. Cl-deficient animal (μM./hr.)
49	0·14	6·1	3·8	3·1
49	0·14	4·9	2·4	1·5
54	0·14	—	7·3	4·9
59	0·14	9·8	7·9	7·3
60	0·14	6·7	7·7	5·0
62	0·09	5·8	3·7	1·3

The fact that the values for net chloride uptake after activation of the chloride-transporting system may approach the values determined for the tracer influx, and that there is a correspondence between the two in their relations to the external concentration suggest that under these conditions the influx may now give a true picture of chloride transport. For example, in one animal shown in Table 6 the influx and net uptake are recorded at two different external concentrations. Neither was increased by increasing concentrations and the influx did not now greatly exceed the sum of the net uptake and the mean loss rate (from Table 1). The final question was whether activation of the chloride-transporting system increased the chloride influx or decreased the fraction of the influx which was involved in exchange diffusion. To answer this, the chloride influx for animals which had been previously salt-depleted was compared with that for the same animals in which the chloride transport had been activated by chloride deficiency. The results of these experiments are shown in Table 7. The chloride influx was not increased in the chloride-deficient animals; in fact, in some of the animals the influx appeared to be somewhat lower than before. The net uptake of chloride in the chloride-deficient animals now accounted, on the average, for 70% of the influx.

These results are clearly consistent with the view that whereas in the salt-depleted animal a large proportion of the chloride influx is involved in a Cl–Cl exchange reaction, activation of the chloride system involves a switch over to useful transport whereby the inwardly transported chloride ions are exchanged for some other anion and hence a net transport of chloride takes place.

(*f*) *The nature of the chloride-transporting mechanism*

We have seen that, after activation of the chloride-transporting system, chloride uptake takes place from KCl in the absence of sodium and without an equivalent uptake of potassium. It follows therefore that the transported chloride ions must be exchanged for some other anion. The mechanism is therefore basically one of ion exchange, as was found to be the case for sodium transport (Shaw, 1960*a*), and the question of the nature of the exchange naturally arises. This has not been completely settled. A partial answer comes from the use of one of the methods devised for the study of the sodium exchange mechanism (Shaw, 1960*a*). Chloride-deficient animals were placed in a potassium chloride solution and a sample of this solution was passed through a strongly acid ion-exchange resin (Amberlite IR–120), the conductivity of the effluent being recorded. Net uptake of chloride proceeded, and when the animal had practically removed all the chloride from the solution another sample was passed through the resin and the conductivity recorded. This gave a measure of the total number of strong acid radicles present in the external solution before and after the chloride absorption. The measurement of the conductivity of another sample of the water taken at the end of the experiment and passed through a strongly basic ion exchanger (Amberlite IRA–400) indicated the extent to which cations in the solution had been absorbed. The results of these experiments are presented in Table 8. They show that no cation absorption had taken place and at least 75 % of the absorbed chloride was exchanged for an anion which does not form a strong acid. This was most probably bicarbonate but these experiments do not exclude an exchange with hydoxyl ions (which would be converted to bicarbonate by CO_2 excreted by the animal) or with the anion of some other weak acid.

Table 8. *Anion exchange during chloride uptake from* KCl *solutions by chloride-deficient animals*

Specimen no.	Initial conductivity of external soln. (μmhos.)	Initial conductivity of soln. through acidic exchanger (μmhos.)	Initial Cl conc. (mM./l.)	Final Cl. concn. (mM./l.)	Final conductivity of soln. through acidic exchanger (μmhos.)	Final conductivity of soln. through basic exchanger (μmhos)
67	14·5	39	0·16	0·015	9·7	27·5
68	15·5	42	0·16	0·015	10·0	29·5
16	15·0	40	0·16	0·008	11·0	31

The activated chloride-transporting system is clearly quite independent of the sodium-transporting system although it may operate alongside it. The independence of the two mechanisms is further confirmed by the action of inhibitors on the two systems. These effects will be considered in detail in a later paper and it is sufficient to mention here that some inhibitors, such as 5% CO_2 in air, inhibit both sodium and chloride transport probably by depressing the general metabolism of the transporting cells. On the other hand, there are other inhibitors, such as ammonium ions, which greatly reduce sodium transport (Shaw, 1960*b*) but have no effect on activated chloride transport. The effect of these two inhibitors on chloride uptake by one individual is illustrated in Fig. 6.

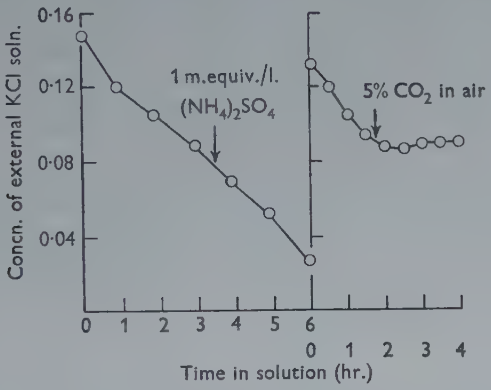

Fig. 6. The effect of 5% CO_2 and of ammonium sulphate on the net uptake of chloride from potassium chloride by a chloride-deficient animal.

Final confirmation of the independent nature of chloride transport in the chloride-deficient animal comes from a demonstration of its active nature. Ussing (1949*b*) showed that if an ion moves across a membrane by diffusion alone and the net flow of water across the membrane is small, then

$$\frac{M_{\text{in}}}{M_{\text{out}}} = \frac{a_0}{a_i} e^{zFE/RT},$$

where M_{in} and M_{out} are the influx and outflux of the ion respectively, a_0 and a_i are the outside and inside ion activities, and E is the potential difference across the membrane. Although this expression is not valid if exchange diffusion forms part of the flux, where there is a net uptake of the ion then it is true that both sides must be greater than 1. Hence E must be greater than

$$\frac{RT}{zF} \ln\left(\frac{a_i}{a_0}\right).$$

For an external chloride concentration of 0·3 mM./l. this expression is approximately equal to +161 mV. Measurements of the potential difference across the surface membrane of the animal were made during activated chloride uptake. The animal was placed in a dish containing a 0·3 mM./l. potassium chloride solution, the depth of which was not sufficient to cover the dorsal part of the carapace. By means of a dental drill a small hole was made through the carapace into the dorsal blood sinus just to one side of the heart. The end of the NaCl bridge from one of the calomel electrodes was inserted through this hole. The other electrode was in the external solution. The results of these measurements are shown in Table 9. In all cases the measured potentials were negative with respect to the blood and thus the potential gradient was in the opposite direction to that required for passive diffusion of the ion. Further, in two cases 5% CO_2 in air, which has been shown to reduce chloride uptake, also reduced the potential difference very markedly. Although it may be argued that an accurate measurement of the potential difference across a transporting membrane can only be made with electrodes placed close to the inner and outer surfaces of the membrane, the fact that the measured potential is of the opposite sign from that expected for passive movement seems to leave little doubt that active transport of the chloride ion must be involved.

Table 9. *The potential difference between the blood and the external solution during net chloride uptake from* 0·3 mM./l. *potassium chloride by chloride-deficient animals.*

Specimen no.	Potential difference (mV.)	Potential difference after bubbling 5% CO_2 in air through ext. soln. for 15 min. (mV.)
64	−26	—
66	−47	−10
67	−41	− 7
68	− 5	—
70	−22	—

DISCUSSION

The results presented in this paper demonstrate conclusively that chloride uptake in the crayfish may be brought about by a process which is quite independent of simultaneous sodium uptake. The facts on which this conclusion is based can be summarized as follows. (1) The chloride influx is independent of the presence of sodium except if sodium is present in high concentrations, (2) during activated chloride transport net chloride uptake takes place when sodium influx is low and a net loss of sodium occurs, (3) net uptake of chloride takes place in the absence of cation uptake and involves an exchange with other anions, probably bicarbonate, (4) net chloride uptake is unaffected by specific sodium transport inhibitors such as ammonium ions, and (5) it takes place against an electrochemical gradient.

In addition to being independent of sodium transport the chloride-transporting system has a number of characteristics which distinguish it from the sodium system. The relation between chloride transport rate and the external concentration has already been noted—a high rate of transport is achieved at a much lower external concentration than in the sodium system (see p. 566). Another important difference between the systems is in the mode of their activation. It was shown previously that the sodium system was activated by salt-depletion and that this led to an increase in the sodium influx (Shaw, 1959). In the chloride system activation is brought about, not by salt-depletion, but under conditions where the animal is deficient in chloride but not sodium. In this system activation does not bring about an increase in chloride influx but serves to switch the mechanism from a predominantly Cl-Cl exchange to a useful exchange of chloride for some other anion.

The fact that sodium transport is activated by total salt-depletion, whereas chloride transport requires a reduction in chloride concentration relative to sodium to activate it, suggests that sodium uptake is the process which is primarily responsible for setting and maintaining the total concentration of the blood. Chloride uptake may be considered as secondary to sodium uptake since it depends on differences between sodium and chloride concentrations in the blood and, therefore, serves to bring the blood chloride level up to that of sodium, once the sodium level has been set.

SUMMARY

1. The mechanism of chloride uptake in the crayfish, *Astacus pallipes*, has been investigated.

2. In the absence of chloride uptake, chloride is lost from the animal at a mean rate of 0·8 μM./hr./10 g. body weight. This is about 72% of the sodium loss rate.

3. In salt-depleted animals, chloride balance can be maintained at a minimum external concentration of 0·028 mM./l. This is lower than the corresponding value for sodium.

4. In salt-depleted animals, chloride is taken up from sodium chloride solutions above the equilibrium concentration, but at about one-third of the rate of sodium uptake. From potassium chloride solutions the net uptake of chloride is slight or non-existent. The addition of sodium increases the rate of net chloride uptake from KCl.

5. The chloride influx is always high and is similar from sodium and potassium chloride solutions. The influx is much higher than expected from measurements of loss rate and net uptake. Up to 90% of the influx may be accounted for by exchange diffusion.

6. Activation of the chloride uptake mechanism can be brought about if the animal is made deficient in chloride only. Chloride influx is not increased but the rate of net uptake may now be high and account for 70% of the influx.

7. Net uptake of chloride by chloride-deficient animals takes place against an electrochemical gradient and chloride is exchanged for other anions, probably bicarbonate.

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THE PHYSICAL BASIS OF ASTRO-NAVIGATION IN BIRDS: THEORETICAL CONSIDERATIONS

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INTRODUCTION

The experiments reviewed by Matthews (1955) and Kramer (1957, 1959) provide strong evidence that homing and migrating birds can use observations of the sun or stars for orientation. In the present paper the possibility of astro-navigation by birds is re-examined from a theoretical standpoint. Revised interpretations of some published experiments are put forward, and suggestions are made for new ones. The paper is concerned with homing rather than migration.

GENERAL PRINCIPLES

Only one quantity need be determined by a bird to enable it to home—the direction of home, expressed as the angle between the required direction and some reference direction. The possibility of an internal directional reference such as a compass has been eliminated by Matthews (1955), so in practice the home direction must be referred to some object which the bird can see. Matthews has also discounted any possibility of obtaining a direct bearing on home from a distance, so the problem resolves itself into that of determining position on the earth; the required bearing must then be deduced by comparing the position so found with the known position of home.

If the earth is considered to have a two-dimensional spherical surface, then the specification of a position on it requires two quantities. The latitude and longitude of a point provide one example of such a pair of quantities, but there is an infinite number of other possible ways of specifying the same point. Each quantity specifies a line, and where the two lines cross is the point in question. Any pair of lines can be used for this purpose, provided that their course over the surface of the earth is known, and that they do not cross in too many places, which gives rise to ambiguities.

To obtain one such position line one observation must be made, either on a landmark or on a celestial object; thus to obtain the two position lines needed to specify a point, two mutually independent observations must be made. If the observations to be made are specified, then a chart of the corresponding position lines can be drawn up, and the accuracy with which they can be plotted, for a given accuracy of observation, can be calculated.

REQUIREMENTS FOR A HYPOTHESIS

A hypothesis of bird navigation must state what two quantities the bird is supposed to be measuring. It must be shown explicitly how the position may be deduced therefrom, and what errors of position will result from given errors of observation. Before experiments are designed to test it, the hypothesis must be examined to make sure that the answers to the following questions are in the affirmative:

(1) Is it physically possible to make the proposed observations under all conditions in which birds are known to navigate?

(2) From the known or estimated performance of the sense organs of birds, is it reasonable to suppose that the measurements could be made to the required accuracy?

(3) Does the hypothesis fit the experimental evidence already available?

Matthews (1955) has demolished all previous hypotheses on one or other of these grounds, and it remains to examine his own.

MATTHEWS'S HYPOTHESIS

Matthews (1953) suggested that the bird should watch the sun for a short time and extrapolate the observed portion of its arc to the meridian. The predicted meridian altitude would then be compared with the remembered meridian altitude seen when the bird was last at home, giving the difference of latitude between home and the release point. The predicted time of meridian transit would be compared with the corresponding time at home, giving the difference of longitude.

Matthews does not state explicitly what two quantities the bird is supposed to be measuring, but it appears from his diagram (Fig. 1) that he has in mind (1) the sun's altitude; (2) the angle at which the sun is moving across the sky, i.e. the change of altitude for a measured small change of azimuth.

These proposed observations may now be scrutinized as suggested above.

Physical possibility

(1) It is physically possible to measure the sun's altitude within certain limitations. These will be discussed later.

(2) Measuring the angle at which the sun moves up the sky involves measuring increments both of altitude and azimuth. If it is possible to measure altitude then it is possible to measure a change of altitude. A change of azimuth can be measured by reference to some object which does not move sideways, or by some internal standard of direction of great stability; the labyrinth is not suitable for this purpose since it measures angular accelerations, not velocities, and can therefore stabilize angular velocity but not position (Löwenstein & Sand, 1940). In any case birds very often fly round in circles whilst orienting, and therefore cannot have their eyes stabilized in azimuth. The change in azimuth can therefore be measured only by reference to a fixed object, when the bird is stationary on the ground. Over the sea it is not physically possible to measure it at all without some kind of compass.

However, birds do navigate over the sea (Mazzeo, 1953; Kenyon & Rice, 1958; Tickell, 1960). Hence Matthews's hypothesis as it stands is untenable.

Theoretical fallacy

Relying on a measurement which is not physically possible is enough to dispose of the hypothesis, but there is an important fallacy in the argument which should be mentioned at this point.

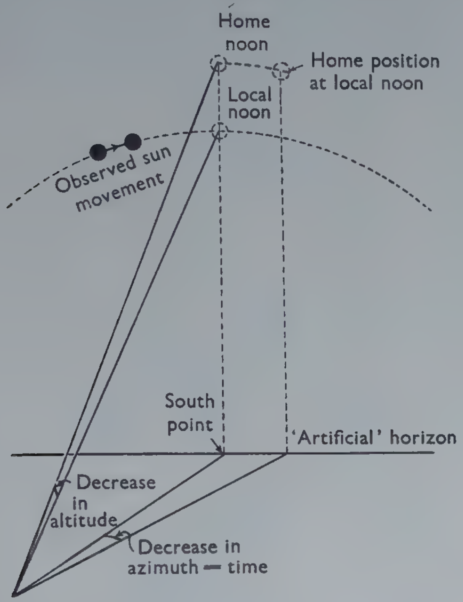


Fig. 1. Diagram illustrating Matthews' hypothesis of sun navigation. Release to north and west of home. From Matthews (1953).

When Matthews considers the errors of position which would result from errors of observation on the part of the bird, he assumes that they will appear as an error in latitude (due to predicting the noon altitude incorrectly), and an error in longitude (due to an error in the predicted time of transit). Thus he assumes that an observation of the quantities mentioned above at any time of day is equivalent to a meridian altitude observation.

Now it is not valid to assume that one kind of observation is equivalent in all its properties to some other kind of observation unless this can be explicitly proved. In Matthews's (1953) paper it is not shown how the meridian altitude data may be calculated from the original observations. He appears to have substituted the meridian altitude in the discussion in place of the observations which the bird is actually supposed to make, because its properties are easier to analyse. His discussion is therefore valid only in the special case where the bird has actually observed a meridian altitude.

MODIFIED SUN-NAVIGATION HYPOTHESIS

The following hypothesis will now be developed. It is supposed that on release the bird measures (1) the sun's altitude; (2) the rate of change of the sun's altitude, i.e. the increment of altitude for a measured small interval of time.

On this hypothesis the bird is not required to be able to tell whether the sun is going from left to right or from right to left. Kramer (1957) suggested this basic idea, but rejected it as impracticable without developing it further.

It has been remarked above that to every observation there corresponds a position line. The properties of the lines corresponding to the above observations will now be discussed.

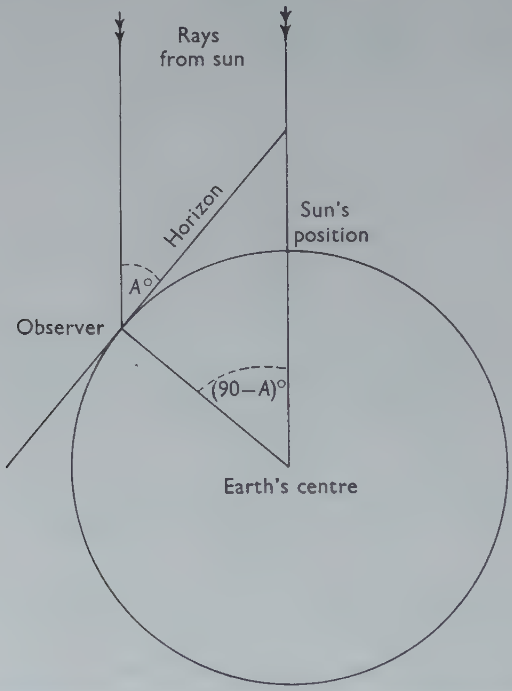


Fig. 2. If the observer finds the sun's altitude to be $A°$, he must be distant $(90-A)°$ from the point where the sun is overhead (sun's position). The meaning of 'distance' expressed in angular measure is evident from the diagram. The observer's position must therefore be somewhere on a circle whose pole is the sun's position and whose radius is $(90-A)°$.

Altitude

The position line corresponding to a timed observation of altitude is known as a Sumner circle, and is discussed in books on navigation. It is a small circle whose pole is the sun's position at the time of the observation and whose radius is $(90–A)$ degrees, where A is the observed altitude.

An error in the measurement alters the radius of the circle, making the observer appear too near or too far away from the sun, according as the measurement is too large or too small.

Rate of change of altitude

The properties of lines of equal rate of change of altitude have been discussed by Hervieu (1960). The position line corresponding to a timed measurement of rate of change of altitude is always a great circle, which passes through the sun's position and its antipodes. The set of these circles thus bears the same relation to the parallels of equal altitude as do circles of longitude to those of latitude, but the poles of the system are at the sun's position and its antipodes instead of at the geographical poles.

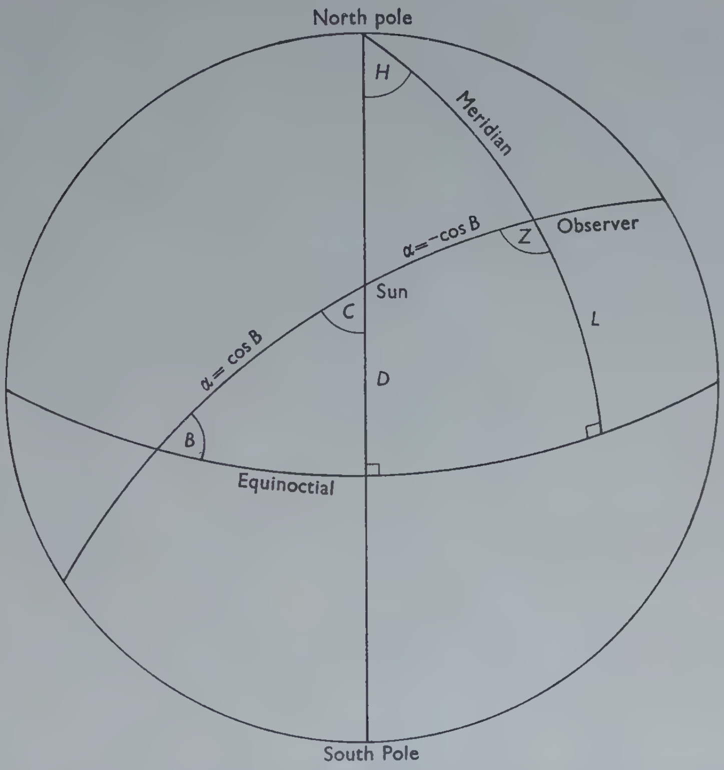


Fig. 3. This diagram represents the celestial sphere with the observer's position and meridian projected on to it. The equinoctial is the projection of the equator on the celestial sphere, and for practical purposes may be regarded as the same thing. The sun is shown with a declination (celestial latitude) $D°$ north of the equinoctial, the observer's latitude being L. Z is the sun's azimuth as seen by the observer, and H is the hour angle—the angle between the observer's meridian and that of the sun, which continually changes as the earth rotates. The line of equal rate of change of altitude (α) is a great circle passing through the sun's position.

An error of measurement causes the position line to rotate about the axis joining the sun to the earth, and thus results in an error of position at right angles to the sun's direction. The magnitude of the error corresponding to a given error of measurement is highly variable and will be discussed below.

Navigational lattice

When the lines corresponding to various values of observed quantities are constructed, a lattice results. Fig. 4 is drawn for the special case of equinox, where the sun's position is over the equator; that is, its declination (celestial latitude) is zero.

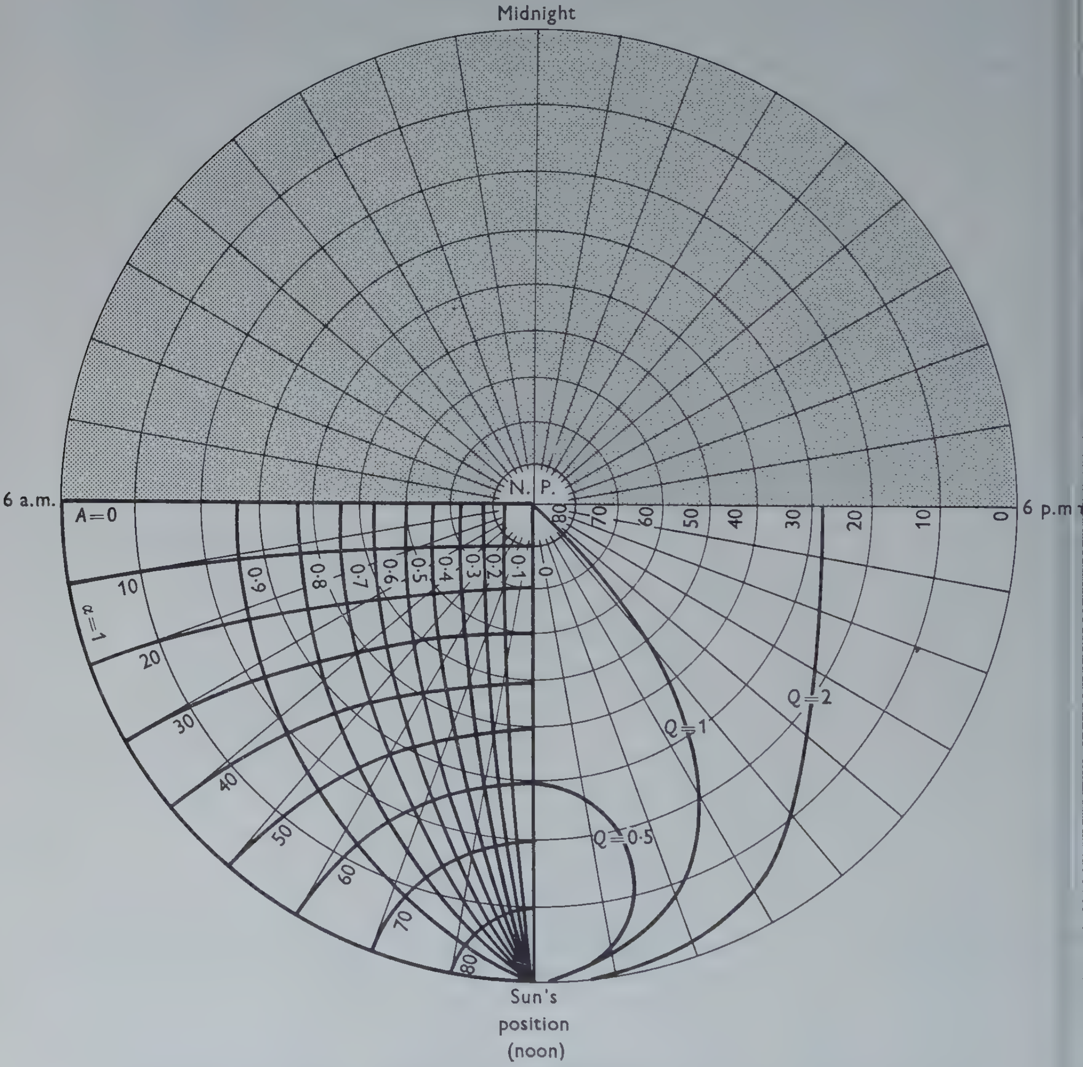


Fig. 4. Stereographic projection of the northern hemisphere, North Pole in the middle. The sun's declination is zero, that is, the season is equinox, and the sun is over the equator. The sun is at bottom centre. Circles of equal altitude are parallel small circles round the sun, resembling parallels of latitude, only with the sun instead of the North Pole as pole. The great circles radiating out from the sun's position are circles of equal rate of change of altitude. In this case the maximum possible value of 1 degree change of altitude for 1 degree hour angle is attained along the equator. Along the sun's meridian (noon) the rate of change of altitude is momentarily zero. The lines on the right-hand side of the diagram join points where the error of position for a given error of rate of change of altitude is constant (see p. 586).

Lines of equal altitude form parallel circles about the sun's position, and the lines of equal rate of change of altitude radiate out from the sun's position, crossing the altitude lines everywhere at right angles. The up-sun direction (i.e. towards the sun) stands in the same relation to this lattice as does north to the latitude–longitude lattice.

If it be asked how the bird can convert an observation of altitude and rate of change of altitude into latitude and longitude, the answer is that no such step is necessary. The observations already specify a point on the lattice, but the lattice is fixed relative to the sun's position instead of being fixed relative to the earth. To obtain the required direction in which to fly the bird must then compare its observations with the values which altitude and rate of change of altitude would have at the same time at home (these are the 'home co-ordinates' in the same lattice). The analysis is extremely simple and proceeds as follows:

(1) If the sun is too low, fly towards it, if too high fly away from it.

(2) If the rate of increase of altitude is too high, fly to the left of the up-sun direction, and vice versa.

South of the dotted line in Fig. 5 the angle laid off would be of the same magnitude but in the other direction. The magnitude of the angle will be given in the section on errors.

The notion of extrapolating the observed portion of the sun's arc to the meridian is rejected for three reasons. First, it is redundant, in that the bird can learn nothing new merely by rearranging the information which it already has. Secondly, it gives a misleading idea of the nature of the information which can be obtained from an ex-meridian observation, and of the magnitude and direction of the errors which would result from errors of measurement—these errors will be further discussed. Finally, it makes the process of extracting the homing direction from the initial observations appear much more complicated and difficult than it need be.

Previous writers have generally assumed that a certain minimum 'orientation time' must elapse after release before the bird can 'get its bearings'. On the present hypothesis, if a clear visual horizon were available, there is no reason why a difference of altitude should not be perceived and acted upon more or less immediately. If the displacement were predominantly across-sun, however, the bird would be expected to need a little time to measure the rate of change of altitude. The orientation time should thus depend on the angle between the sun's direction and the homing direction, and also on the magnitudes of the differences in the co-ordinates to be measured and the clarity of the horizon.

Matthews's concept of remembering the sun's motion as observed at home is retained, as this avoids the necessity for any nautical almanac information being built into the bird. In the case of migration without prior experience, however, some such assumption might be necessary. The feat of memory required may appear a prodigious one, but it should be remembered that the movement of the sun has only three degrees of freedom provided the observer is at rest, and three numbers are sufficient to specify its motion completely at a given point.

The problem of using the stars for orientation is easier than using the sun. Altitudes of two different stars can be used simultaneously in the way described above, and there is then no need to measure rates of change. The planetarium experiments of Sauer & Sauer (1955) showed that birds can orient by a star pattern which is not moving, and this proves that rates of change can be dispensed with.

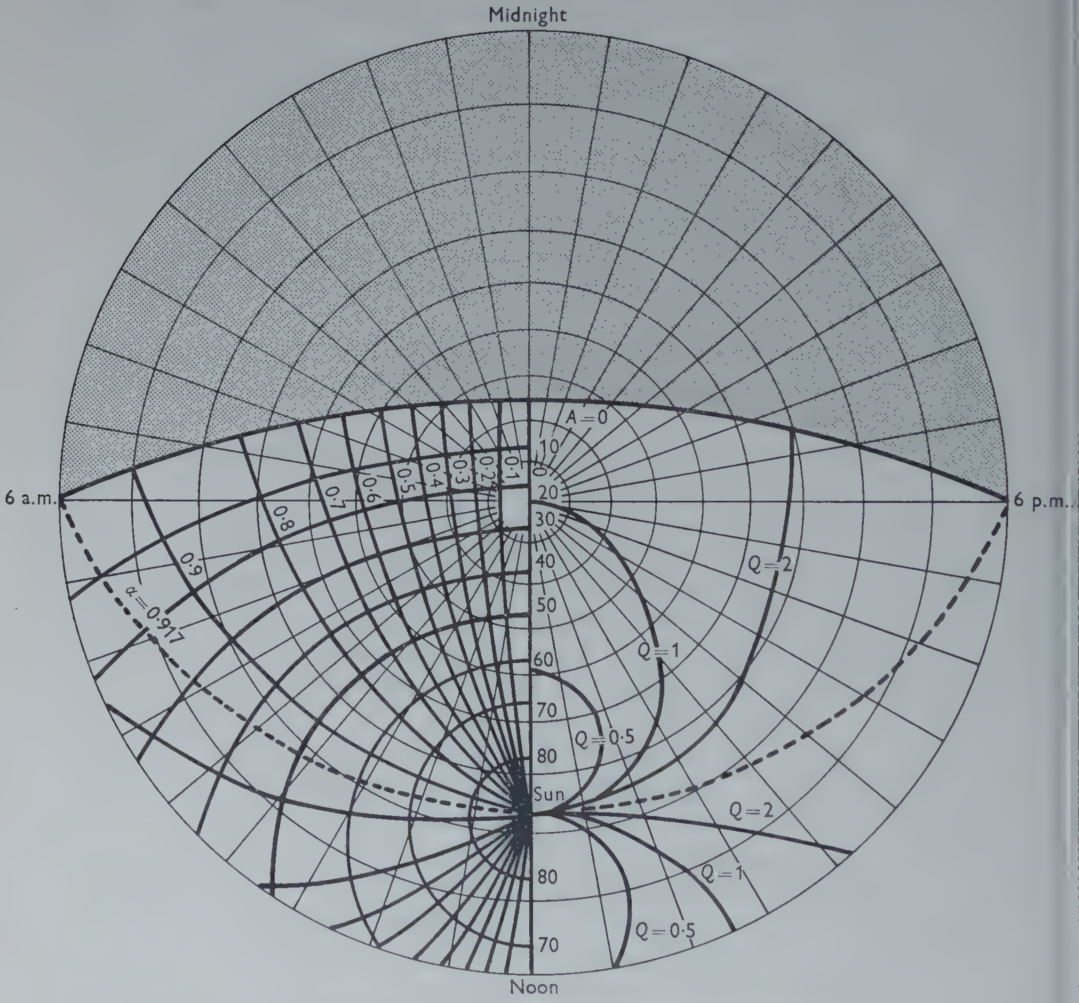


Fig. 5. As Fig. 4, but at northern midsummer; the sun's declination is now $23\frac{1}{2}°$ N. The maximum rate of change of altitude is now 0·917, and is attained along the line passing through the sun's position and crossing the equator at 6 a.m. and 6 p.m. (dotted line). The lattice south of this line is a mirror image of that north of it.

Fig. 5 shows the lattice obtained in the northern hemisphere at summer solstice. The sun has now moved to $23\frac{1}{2}°$ N., and the whole lattice has tilted up to follow it. The maximum value of rate of change of altitude is now only 0·917, whereas at equinox (Fig. 4) it has the maximum possible value 1 along the equator, meaning 1 degree change of altitude for 1 degree rotation of the earth.

The circle of maximum rate of change of altitude passes symmetrically through the sun's position, crossing the equator at 6 a.m. and 6 p.m. It can be seen that to the south of this line an ambiguity can arise, since for every point north of the line there is another with identical co-ordinates south of it. Going round a circle of equal altitude one would pass the rate lines in reverse order on the two sides of the line; hence a bird released south of the line, and under the impression that it was north of it, might be expected to lay off the correct size of angle from the sun's direction, but the wrong way. It would thus not home to the southern point corresponding to its northern home, but would end up flying directly away from it, creating an ever-increasing discrepancy in the rate of change of altitude. This effect could be used by a trans-equatorial migrant to identify which hemisphere it was in.

EXAMINATION OF THE HYPOTHESIS

The hypothesis will now be examined under the heads listed above, though they will not be kept strictly separate.

Possibility and feasibility of measurements

(1) The altitude of a celestial object is the angle subtended at the observer between the object and the observer's horizon. To measure it the observer therefore has to locate both the object and the horizon within the required limits of accuracy. Locating the horizon is much the more difficult proposition.

It is physically possible to identify the horizontal (or vertical) in two ways, by reference to gravity, or visually. The use of gravity is feasible when the bird is stationary on the ground. The kind of co-ordination of eye and labyrinth which would be required has been investigated in man by Neal (1926) and Noble (1949), both of whom found that a subject in the dark could set a luminous line to the vertical with an accuracy better than 1°. If the bird could improve on this by a factor of 5 an accuracy of 12 nautical miles could be obtained from altitude observations. It is not therefore unreasonable that pigeons kept by Kramer in small aviaries with an irregular skyline, and not allowed exercise flights, should still be able to orient when released away from home; the movements of the sun at home could have been observed with reference to gravity.

Gravity can be used in flight as a vertical reference, provided accelerations can be measured by some sense independent of the one which is measuring gravity. In smooth air over land an airspeed sense combined with rate of turn obtained visually might serve (rate of turn cannot be measured by the labyrinth). In turbulent air the problem is very difficult; it is conceivable that the bird could obtain a sufficiently accurate horizon by 'smoothing out' lateral accelerations as is done in aircraft artificial horizons. The integration would have to be done neurally as no sense organ is known which could do this.

If a clear visual horizon were available, the eyes of many birds have certain features which would make them very suitable for locating it. A number of writers have commented on the presence of a ribbon-like area of acute vision in the retinae of many birds (not pigeons). Duijm (1958) has measured the inclination of this *area* to the axis of the bill in four species, and shown that in the normal head position of each the *area* is held approximately horizontally, so that the image of the horizon might be expected to fall upon it.

If the visible horizon should be below the horizontal plane, as when seen by a bird flying high above the sea, or above it, as could happen if the bird were flying below the top of a haze layer, the bird could still level its head laterally by comparison of the images in the two eyes. If the discrepancy in the horizon is the same on both sides, then the bird's head will be level when the displacement of the image is the same in both eyes. If the horizon is indistinct, as it very often would be over land, the bird might be expected to scan all round it by flying round in a circle, in order to arrive at a compromise. Over uneven country the presence of a low haze layer above which the bird could climb would greatly facilitate determination of the horizon. It may be expected that over land every ground position would produce an apparent horizon tilted in a characteristic manner as compared to the true one—the 'electric brae' in Ayrshire, where cyclists appear to freewheel uphill, is an example of a landscape which produces a gross illusion of this kind clearly perceptible to man. The effect of a slight tilt on orientation will be considered below.

At night the horizon is generally difficult to locate at sea level, but often becomes more distinct at higher altitudes. In some circumstances, e.g. above a temperature inversion in moonlight, a good horizon would be available. Lack's (1960) radar observations of migration across the North Sea showed that most of the birds flew at heights between 2000 and 5000 ft.; up to 10,000 ft. was not uncommon, and one bird was recorded at 14,000 ft.

Evidence that birds do level their heads laterally by comparing the horizon in the two eyes, when a clear visual horizon is available, is provided by the 'palisade' experiment described by Kramer (1959). Pigeons were kept in a small aviary closely surrounded by an opaque palisade so constructed as to cut off the bird's field of view at least 3° above the horizontal. It can be seen from the accompanying revised version of Kramer's drawing (Fig. 6) that if the bird levels its head in the way suggested, large variations in the apparent horizontal will be produced by moving about the cage. This would cause the apparent altitude and rate of change of altitude to vary in an irregular fashion, which might be expected to prevent the bird from determining its home co-ordinates. In fact, birds reared in this way show no orientation when released away from home. Kramer's conclusion that this shows the bird must be relying on clues in the bottom 3° of the sky, which he arrived at by eliminating a number of other explanations, is logically unsound since he had not thought of all the possibilities.

(2) A rate of change of altitude can be measured if an altitude can, but the practical requirements of the problem are rather different. Let us suppose that the bird is flying round in circles, as homing birds commonly do on release, with its head held level on a visual horizon. The sun's image will then trace a line across

the retina. As the bird enters another circle, or turns back, the sun's image will trace another line on the retina. The sensory problem is to determine the distance separating the two lines, and the time interval between them.

The accuracy with which the rate of change of altitude would have to be measured to obtain an accuracy of 12 nautical miles, taking a value of $Q = 2$ (explained below) would be about $1\frac{1}{2}$ sec. of arc per minute of time.

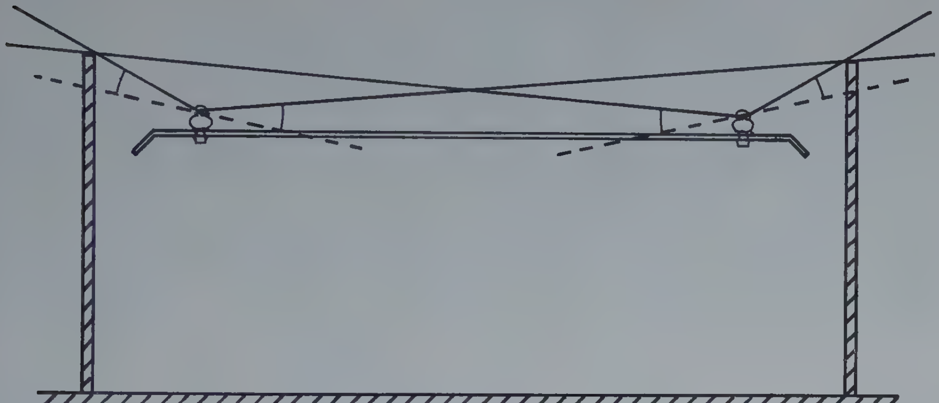


Fig. 6. Redrawn from Kramer (1959). Pigeon in cage surrounded by an opaque palisade, which is higher than the bird's eye level. If the bird levels its head by comparing the horizon on the two sides, its estimate of the horizontal will be tilted different amounts in different parts of the cage. Dotted lines show apparent horizontal at two different points.

The minimum width of line which the human eye can detect (and presumably in principle locate) is around 30 times less than the minimum separation of two points required to make them separately visible (Hartridge, 1956). Pumphrey (1948) estimates the latter measure of acuity as 10 sec. of arc for the general run of bird eyes, so the ability to locate a line may be expected to be in the region of $\frac{1}{3}$ sec. of arc. The probable error in the difference between two lines would therefore be of the order of $\frac{2}{3}$ sec. of arc. In other words, the bird would have to take two observations separated by an exactly measured time of about $\frac{1}{2}$ min. to obtain the required accuracy of rate of change. If observations were made at more frequent intervals and averaged, the accuracy could of course be improved, or the observation time shortened.

The short-term time sense involved in the measurement could be entirely intrinsic in the retina. Hartline (1938), recording from single fibres in the optic nerve of the frog, found certain 'off' units which continued firing at a gradually decreasing rate for several seconds after the cessation of a stimulus. If the avian retina is supposed to contain such cells, which either go on firing or undergo some change such as an altered threshold which decays in a predictable manner over several seconds, these could be used both to mark the course of the sun's last traverse of the retina, and to give an indication of the time which has elapsed since it passed. The sun's image, being a vigorous form of stimulation, is especially suitable for producing large changes which would take some time to decay.

In the above calculation it is assumed that the short-term time sense is perfectly accurate, but an error in it would have the same effect as an error in locating the track of the sun's image.

Whether birds can actually make these measurements in practice is a matter for experiment. The writer is only concerned to show that the performance required is physically possible and quite reasonable on the information available.

THE EFFECT OF ERRORS OF MEASUREMENT

There a four sorts of error which would affect the orientation of a bird homing by the method proposed in this paper. These are in the determination of (1) the horizontal, at home or at the release point, (2) the sun's altitude, (3) the rate of change of the sun's altitude, (4) the time.

Each of these may be considered to give rise to a displacement of position in a particular direction. An error in determining the horizontal at home gives rise to an apparent displacement of home, whereas the others produce apparent displacements of the release point.

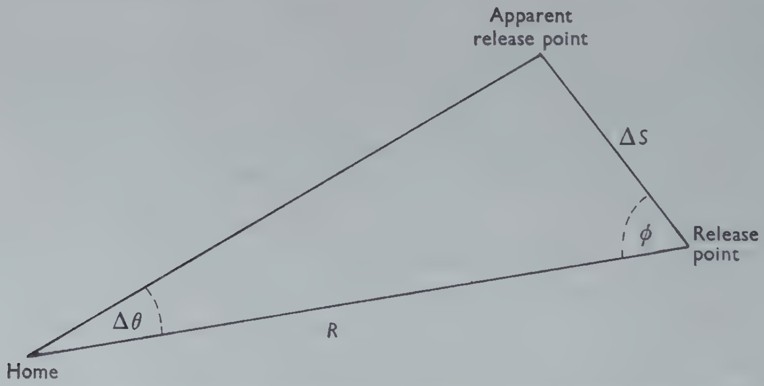


Fig. 7. If the bird makes an error of observation on release, it will appear that, instead of being at the release point, it is at some other point, a distance ΔS away, at an angle ϕ to the homing direction. This will result in an error $\Delta\theta$ in the direction of orientation. The relationship between these quantities and the true homing distance R, over short distances for which the earth's surface can be regarded as a plane, is given in the text.

Suppose that owing to some kind of error the release point appears to be displaced a distance ΔS at an angle ϕ with the true homing direction (Fig. 7). If the distance from home to the release point is R, an error $\Delta\theta$ in the homing direction will result. Its approximate magnitude, for short distances over which the earth's surface can be regarded as a plane, is given by

$$\sin \Delta\theta = \frac{\Delta S \sin \phi}{\sqrt{(\Delta S^2 + R^2 - 2R\Delta S \cos \phi)}}. \tag{1}$$

An apparent displacement of home calls for an adjustment of R and the homing direction from which ϕ is reckoned, after which the calculation for displacement of the release point is the same as before.

The displacements ΔS resulting from various kinds of error will now be considered. The formulae presented all give ΔS in nautical miles (n.m).

Error in determining horizontal

If the apparent horizon is tilted towards or away from the sun the altitude would be affected but not the rate of change of altitude, whereas if it is tilted at right angles to this direction the rate of change of altitude alone will be affected. Thus over the course of a day both altitude and rate of change of altitude are altered to various degrees. The apparent motion of the sun which results is the same as would occur at that point where the true horizon is parallel to the false one being used. Thus a horizon tilted 1° with its north-western side downwards would give rise to motions of the sun appropriate to a point 60 n.m. to the north-west, where the earth's surface is parallel to the false horizon being used.

The apparent position of home could be shifted in this way either by accident or on purpose (this possibility will be further discussed). Also, a false horizon at the release point would cause an error of orientation; if the error in the horizon is $\Delta\xi$ min., then $\Delta S = \Delta\xi$ n.m. in the direction of tilt.

Tilted horizons produced by the surrounding landscape seem the most likely explanation for the variation in orientation at the various release points used by Walraff (1959) around Wilhelmshaven. Each point seemed to produce a definite orientation in one direction, but not always towards home. There were two points from which the birds generally departed in the direction away from home, although there were certain days when the birds oriented instead in the right direction; it is not recorded whether there was a low-level temperature inversion on these days, which would have produced a good horizon more or less independent of the landscape.

The releases around Durham, North Carolina, described by Pratt & Thouless (1955), were made from points in a variety of landscapes, including mountains, where illusions might be expected to occur most readily. In one experiment birds from two different lofts were released at a point between them: those from one loft (A) oriented strongly and homed well, whereas those from the other (B) flew in circles for a long time and homed very badly. This suggests that the apparent horizon at the release point was parallel to the true horizon at loft B, giving enhanced orientation indications to the birds from loft A, whereas those from loft B would be able to detect no difference from home.

One case in which orientation affected by overcast was observed at a range of only 7½ miles could be due to a horizon which happened to be tilted in the right direction.

The so-called 'direction effect' (Kramer, Pratt & von St Paul, 1956) could also be due to this cause. It is found in Wilhelmshaven and Durham, N.C. (though apparently not in Cambridge or California), that pigeons orient better from the south than from the north; east and west being intermediate. This could easily be due to a fortuitous variation in the properties of the release points used.

Error in altitude

An error ΔA in altitude produces an error of the same magnitude on the ground. Thus $\Delta S = \Delta A$ n.m., where ΔA is in minutes. This error appears *up-sun* if the observed altitude is larger than the true one, *down-sun* if it is too small. An error of either the observed altitude or the remembered home altitude will produce this result.

Error in rate of change of altitude

The distance moved for a small change of rate of change of altitude depends on the spacing between the lines of Figs. 4 and 5. These lines radiate out from the sun's position, so their spacing varies along their length and also from one line to the next, as the angular separation between them is not constant.

It is shown in the appendix that

$$\Delta S = 3440\Delta\alpha \frac{\cos A}{\sqrt{(\cos^2 D - \alpha^2)}} \text{ n.m.,} \tag{2}$$

where α is the rate of change of altitude in degrees of altitude per degree of hour angle (1 degree of hour angle = 4 min. of time), $\Delta\alpha$ is the error therein, and D is the sun's declination (celestial latitude).

The displacement is always *across-sun*.

The probable error of observation $\Delta\alpha$ may be assumed constant under constant conditions of observation by the same bird, but the other factor, Q, where

$$Q = \frac{\cos A}{\sqrt{(\cos^2 D - \alpha^2)}},$$

is highly variable. In general, the lower the latitude the more it fluctuates with different seasons and times of day.

Fig. 8 shows the daily variations at four different latitudes, 53½° N. (Wilhelmshaven), 36° N. (Durham, N.C.), 25½° N. (Laysan Island), and 8° N. (Ascension Island is 8° S.). The curves are given for midsummer, midwinter, and equinox at each. It can be seen that the daily fluctuation is greater in summer than in winter, the minimum values of Q being lower and maximum values higher. Also the fluctuations are greater in lower latitudes, the June daily range being 2·6:1 in Wilhelmshaven, 9:1 in Durham, N.C., 70:1 at Laysan, and 40:1 at 8° N. (which, being within the tropics, does not have the greatest fluctuation in June). The maximum values are again higher and the minimum values lower. This is one possible explanation for the finding that performance in general is more erratic at Durham, N.C., than at Wilhelmshaven or Cambridge.

Matthews (1955) and Walraff (1959) report experiments in which releases were continued all day, and poorer performance was observed in the early morning and late afternoon than in the middle of the day. Unfortunately these results cannot be correlated with the graphs of Fig. 8, since the latter refers only to the accuracy of *cross-sun* orientation; in an all-day release from one point the sun's direction is continually changing, and the bird is relying on a varying mixture of up- or down-sun and cross-sun clues.

The value $Q = 2$ used in an earlier calculation was taken from these curves and can be seen to be unduly pessimistic for Germany, and for the middle part of the day at the other places. This can also be seen from the spatial distribution of Q shown in Figs. 4 and 5; the line for $Q = 2$ never comes above about 33° N.

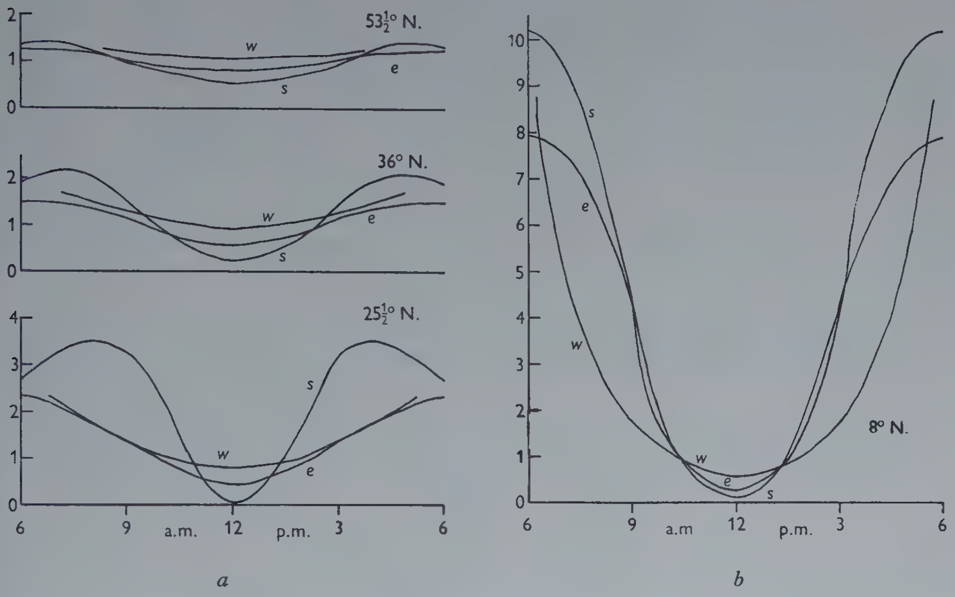
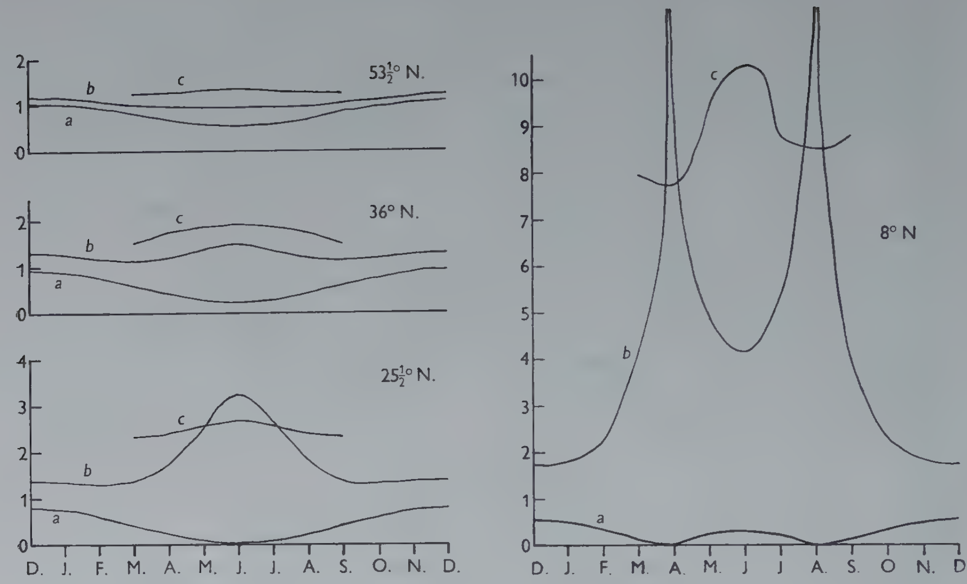


Fig. 8. Variation of Q with time of day at four different latitudes (see text). *w*, midwinter; *e*, equinox; *s*, midsummer.

Fig. 9 shows the seasonal fluctuations for the same four places, the three curves for each now being drawn for different times of day. It can be seen that the shape of the seasonal curve is very sensitive to time of day, especially at Laysan, where it is more or less inverted between noon and 3 hr. away from noon. Within the tropics Q goes to infinity whenever the line of maximum rate of change of altitude passes overhead, meaning that along that line there is no change in rate of change of altitude for a small movement in any direction.

The curve given by Kramer (1954) showing homing success better in summer than in winter is again not comparable with the curves of Fig. 9, this time for three reasons: the angle between the homing direction and the sun's direction is not considered; time of day is not taken into account; and, as emphasized by Matthews (1955), homing performance cannot be regarded as a measure of accuracy of position finding, and accuracy of initial orientation only should be included in such curves.

It can be seen that Q must also be taken into account in determining the homing direction from given observations. If the angle between the homing direction and the sun's direction is θ, if A_r and α_r are the sun's altitude and rate of change of



a

b

Fig. 9. Variation of Q at different times of year (see text). Abscissa marked at 22nd of each month. *a*, noon; *b*, 9 a.m. and 3 p.m.; *c*, 6 a.m. and 6 p.m.

altitude observed at the release point, and if A_h and α_h are the corresponding home co-ordinates, then

$$\tan\theta = \frac{(\alpha_r - \alpha_h)}{A_r - A_h} \cdot Q \cdot 3440 \tag{3}$$

This formula is an approximation for short distances, where Q does not alter appreciably over the displacement used, and the earth's surface approximates to a plane.

Error of time

The bird must be able to estimate time in order to compare the altitude and rate of change of altitude which it has measured with those which it would have observed at the same time at home. This is a long-term day-to-day time sense, quite distinct from that needed to measure rate of change of altitude. This error is the only one which is directly related to compass points, since an error of time affects both altitude and rate of change of altitude, and simply results in an error of longitude. Its magnitude is

$$\Delta S = 15 \Delta t \cos L \quad \text{n.m.},$$

where Δt is the error of time in minutes and L the latitude. It appears east if the bird's clock is slow, west if it is fast. The error in orientation is thus zero for releases east or west of home, and maximum for those north or south.

Experiments such as those of Schmidt-Koenig (1958), designed to produce shifts of several hours in the bird's clock, demonstrate that the bird has a clock which can be shifted but do not throw any light on how accurate it is.

SUGGESTIONS FOR EXPERIMENTS

Shifting the horizon

The following experiment should show whether astro-navigation is used at all, though it would not discriminate between one type of observation and another. The bird would be brought up with a false horizon as in Kramer's palisade experiment, but it would be kept in a small cage surrounded by a very large palisade, whose apparent inclination would then be more or less constant. The palisade would be accurately constructed so as to be tilted from the horizontal by a known amount in a particular direction. The bird would then become accustomed to sun's motions corresponding to some other place as explained above, and when released should orient towards the 'false home'.

As an example of the order of dimensions involved, a bird allowed 2 ft. horizontal movement, in the middle of a palisade 50 ft. in diameter and 3 in. above the bird's eye level, could tilt the horizon through about 3 min. by moving about the cage. The best arrangement would probably be to keep a number of birds in an enclosed aviary with a small observation box at the top, the only place from which they could see the sun and the palisade.

If this experiment were found to work it might have practical applications, in that pigeons could be raised in one place which would home to another place.

Discrimination between measurements

To test the hypothesis put forward in this paper, an experiment could be designed to take advantage of the variations of accuracy obtainable from the different measurements. A release point would be chosen to the east or west of home, so that errors of time would produce negligible effects. Choosing a time of year when cross-sun orientation should be good, two groups of birds would be released, one in the early morning (using cross-sun orientation), and one about midday (using up- or down-sun orientation). This would be repeated at a time of year when cross-sun orientation should be poor. The accuracy of up- or down-sun orientation should be constant at any time of year, so the good and poor cross-sun birds could be compared, using the up- and down-sun birds as controls.

Another variant of this experiment could be used to investigate the accuracy of the clock. In this case two release points would be used, one north or south and the other east or west. Conditions would be selected so that cross-sun accuracy should be the same for all birds, so that one group being affected by errors of time and the other not would be the only difference between them.

Unintentional horizon errors

Horizon errors over the land are very difficult to eliminate or allow for. A possible cure for this source of confusion would be to use sea birds breeding on a small island and release them from a boat out of sight of land. The navigational faculty might be expected to be most highly developed in such forms anyway.

SUMMARY

1. Matthews's hypothesis of sun navigation in its original form is rejected on the grounds of physical impossibility of making one of the measurements, and an invalid assumption in the reasoning.

2. It is suggested that the bird should measure the sun's altitude and rate of change of altitude. The position lines corresponding to these quantities are discussed, and the way in which birds might make and use such observations is considered.

3. The sensory performance required is shown to be reasonable.

4. The magnitude and direction of the errors of position resulting from various sorts of errors of observation are discussed in relation to the experimental evidence.

5. Suggestions are put forward for future experiments.

APPENDIX

The equations used in the text could be derived from the theory of lines of equal rate of change of altitude given by Hervieu (1960). The writer's treatment was developed independently, and although it is the same in essentials parts of it are given here since they lead in a simpler and more direct manner to the particular results required for the present purpose.

The terms used are explained in books on navigation, e.g. Cotter (1953), and it is suggested that the reader unfamiliar with this subject should first consult such a work before proceeding further.

The relationship between altitude, declination, latitude and hour angle is

$$\sin A = \cos H \cos L \cos D + \sin L \sin D, \tag{1}$$

where A is the altitude, H the hour angle, L the latitude, and D the declination.

The rate of change of altitude with respect to time is proportional to the rate of change with respect to hour angle, and the latter quantity, represented by the symbol α, is used throughout. Hence, from (1),

$$\alpha = \frac{dA}{dH} = -\frac{\sin H \cos L \cos D}{\cos A}. \tag{2}$$

If α, D and A are given, then (2) becomes a relationship between H and L, specifying a great circle as shown in Fig. 3.

The usual expression for the sun's azimuth, Z (Fig. 3), is

$$\sin Z = -\frac{\sin H \cos D}{\cos A}. \tag{3}$$

Substituting in (2) we have that

$$\alpha = \cos L \sin Z.$$

Now if B is the angle at which the line of constant α crosses the equator (Fig. 3), then it follows from the equations of a right-angled spherical triangle that

$$\cos B = \cos L \sin Z = \alpha. \tag{4}$$

It should be noted that α changes sign as the line passes under the sun, but its magnitude remains the same. It can be seen from Fig. 3 that B can never be less than D, so at any given time of year the maximum value of α is $\cos D$, which occurs along the great circle which passes under the sun and crosses the equator at 6 a.m. and 6 p.m.

Fig. 10 shows the effect of a slight error in α. The great circle still passes through the sun's position, but B is altered slightly.

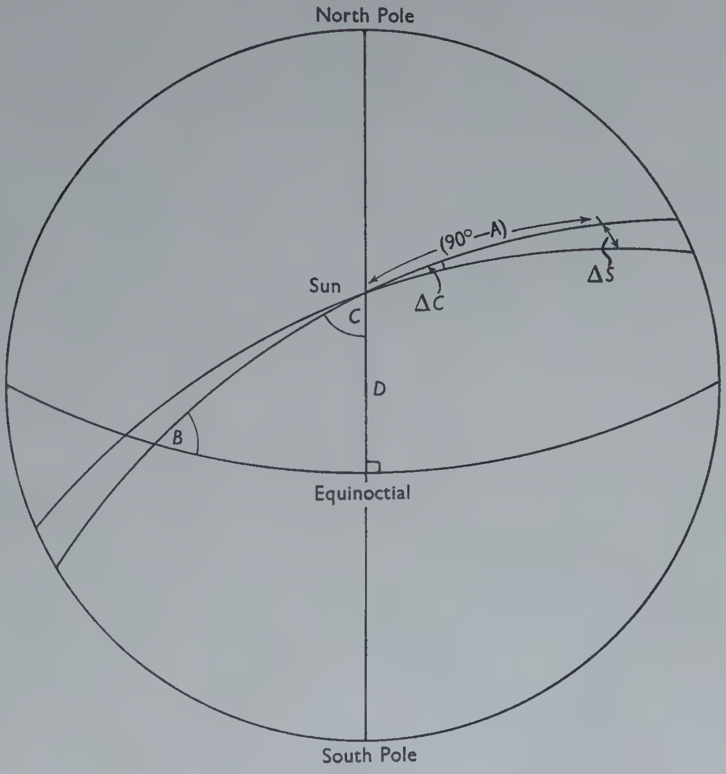


Fig. 10. Relation of error of position to small error of rate of change of altitude (see text).

The error of position ΔS is simply the error in the angle C multiplied by $\cos A$,

$$\Delta S = \Delta C \cos A. \tag{5}$$

From the usual formulae

$$\cos B = \sin C \cos D = \alpha, \tag{6}$$

$$\frac{d\alpha}{dC} = \cos C \cos D. \tag{7}$$

From (6)
$$\sin C = \frac{\cos B}{\cos D},$$

therefore
$$\cos C = \sqrt{\left(1 - \frac{\cos^2 B}{\cos^2 D}\right)},$$

therefore
$$\frac{d\alpha}{dC} = \sqrt{(\cos^2 D - \cos^2 B)} = \sqrt{(\cos^2 D - \alpha^2)}.$$

Hence, for a small error of α
$$\Delta C = \frac{\Delta\alpha}{\sqrt{(\cos^2 D - \alpha^2)}}. \tag{8}$$

From (5)
$$\Delta S = \frac{\Delta\alpha \cos A}{\sqrt{(\cos^2 D - \alpha^2)}}.$$

This gives ΔS in radians, so it must be multiplied by 3440 to convert it to nautical miles.

I am grateful to Dr W. H. Thorpe, F.R.S., for reading the typescript, and making a number of suggestions which have been incorporated in the final version. I am also indebted to Mr D. H. Sadler, O.B.E., M.A., of H.M. Nautical Almanac Office, for drawing my attention to Hervieu's work and for checking the astronomical argument. The work was done during the tenure of a D.S.I.R. Research Studentship.

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STUDIES ON THE EXCHANGE AND REGULATION OF SODIUM IN THE LARVA OF *AEDES AEGYPTI* (L.)

II. THE NET TRANSPORT AND THE FLUXES ASSOCIATED WITH IT

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I. INTRODUCTION

In an earlier paper (Stobbart, 1959) I showed that the steady-state exchange of sodium between the haemolymph of this larva and the medium can be increased roughly six-fold by feeding. The exchange occurs principally through the anal papillae, and there is evidence which suggests that large parts of the influx and outflux of sodium are mediated by a carrier mechanism in the anal papillae.

In this paper I shall describe experiments on the net transport of sodium by sodium-deficient larvae. As before, the aim was to try to throw more light on the processes of sodium exchange and regulation.

II. MATERIALS AND METHODS

Larvae deficient in sodium were produced by rearing from the egg 700–1000 in 500 ml. of glass-distilled water in a hard-glass beaker. They were given a limited amount of food. In order to prevent some of the larvae from growing more quickly than the rest it was necessary to add the food (total amount about 0·3 g.) in small amounts at roughly daily intervals, taking care that some was always available to the larvae. Despite this the variation in growth rate was greater than in larvae reared as described earlier (Stobbart, 1959). The sodium content of the rearing solution was 0·001–0·002 mM./l. and of the haemolymph of sodium-deficient larvae about 30 mM./l.—roughly one-third of that of normal larvae. The sodium-deficient larvae grew about half as fast as normal ones reared as described earlier, and took about 6 days to reach the 4th instar. Though they were rather smaller than normal ones and had greatly hypertrophied anal papillae they were just as active and seemed quite healthy. Sodium-deficient larvae containing labelled sodium were produced by rearing them in distilled water to which a small amount of practically weightless ^{22}Na had been added. The specific activity of the rearing solution varied by reason of the addition of the food. In order to reduce variation caused by this the larvae were collected for the experiments as far as possible at the same time.

Both fed and starved 4th-instar larvae were used in the experiments. The fed larvae were used within a few hours of moulting into the 4th instar and were always given a sufficiency or an excess of food. The starved larvae were kept in distilled water without food for 60 hr. before the start of an experiment. In order to study the net transport of sodium and the fluxes associated with it, sodium-deficient larvae were put into what were effectively infinite amounts of unlabelled or labelled media containing 2 mM/l. Na and of the same composition as those described earlier. The increase in sodium level in the haemolymph was found by flame photometry and, where appropriate, the change in radioactivity in the haemolymph was noted. Where necessary the anal papillae were destroyed by Wigglesworth's method (1933), the larvae being kept in distilled water till scars had formed at the bases of the papillae. All other techniques and symbols were the same as those described earlier (Stobbart, 1959). The lines drawn through the points in some of the figures were drawn by eye unless it is stated to the contrary in the text. The experiments were done at 28° C.

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III. RESULTS

Fig. 1 (which contains data from two experiments) shows the net transport performed by both fed and starved sodium-deficient larvae. In the case of fed larvae the net transport occurs in two phases, both of which are roughly rectilinear. The 1st phase is rapid, the rate of net transport being 55–65 mM./l./hr. At the end of this phase the sodium level has risen to 85–95 mM./l. After this there is a slow significant increase in sodium level. For the two experiments the rates of net transport were 0·544 mM./l./hr., standard deviation = 0·182, $P = 0{\cdot}015$; and 0·177 mM./l./hr., S.D. = 0·0614, $P = 0{\cdot}02$. The transition between the two rates of net transport is rapid. In the case of starved larvae the sodium level rises at a rate of about 10·5 mM./l./hr. and reaches a value of about 110 mM./l. in about 8 hr.; thereafter there is a slight decline to about 105 mM./l. which is the level found in normal larvae (Stobbart, 1959). As the maximum value is approached the rate of net transport decreases rapidly. The net transport by starved larvae in another experiment is shown in Fig. 3A. The increase in sodium level is largely rectilinear, the rate being 6·96 mM./l./hr., S.D. = 0·463, $P = < 0{\cdot}001$. These larvae were for various reasons starved for 98 hr. before entry into the medium and this may account for the slower rate of net transport. The results given above show that feeding increases the rate of net transport by a factor of roughly 5 (neglecting the slow phase of fed larvae).

Fig. 2A shows, for both fed and starved larvae, the outflux of labelled sodium from the haemolymph during net transport. The larvae, which contained labelled sodium, were put into an unlabelled medium at zero time. The flame photometer readings show the initial and final values of the sodium level in the haemolymph. On the basis of evidence presented in Fig. 1 one would expect the rapid phase of net transport in fed larvae to be complete in about 1 hr. and the net transport in starved larvae to be completed in about 8 hr. After these times a steady-state exchange must occur as the sodium level stays effectively constant. The tangents to the radioactivity curves at their points of origin give estimates for the initial outflux of sodium. As the net transport is roughly rectilinear an estimate of the initial influx can be got by adding the initial rate of outflux to the rate of net transport. These estimates are very rough as the initial specifc activity of the sodium

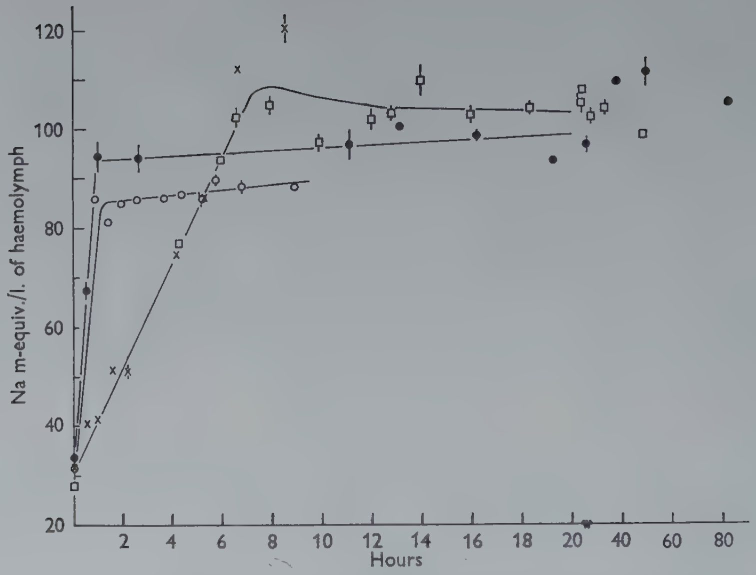

Fig. 1. ● ○ = sodium level (flame photometer) in the haemolymph of fed sodium-deficient larvae. The lines drawn through the points for the slow phase of net transport are the regression lines for sodium level on time. × □ = sodium level (flame photometer) in the haemolymph of starved sodium-deficient larvae. The larvae were reared under sodium-deficient conditions and put into the medium at zero time. The vertical lines show the extents of the standard deviations which are due to variations in the flame photometer.

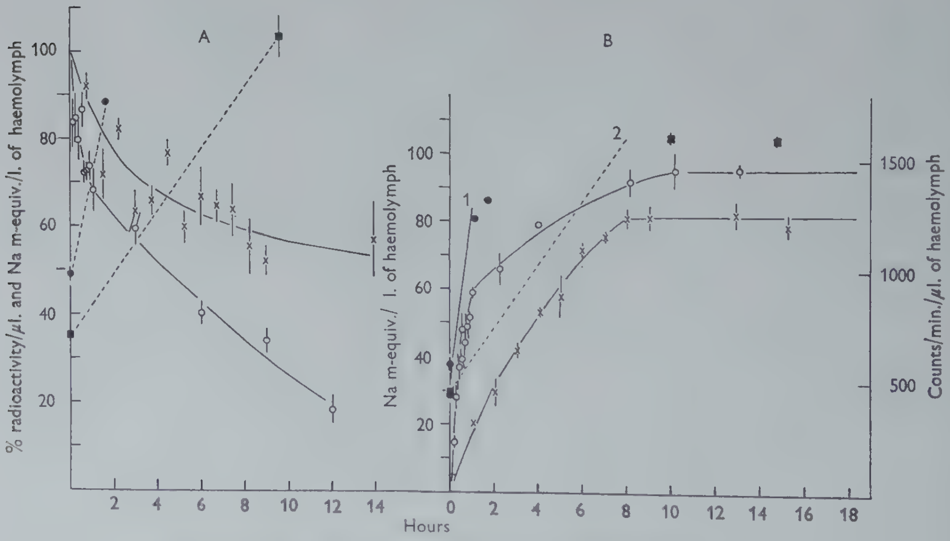

Fig. 2. A: ● = sodium level (flame photometer) in the haemolymph of fed sodium-deficient larvae; ■ = as above but for starved larvae; ○ = outflux of labelled sodium from the haemolymph of fed sodium-deficient larvae—the standard deviations are due to variations between individual larvae, and to the errors inherent in assay of radioactivity; × = as above but for starved larvae. B: ● = sodium level (flame photometer) in the haemolymph of fed sodium-deficient larvae; ■ = as above but for starved larvae; ○ = influx of labelled sodium into the haemolymph of fed sodium-deficient larvae; × = as above but for starved larvae.

in the haemolymph was not found accurately. The estimates are shown in Table 1. Fig. 2 B shows the influx of labelled sodium into the unlabelled haemolymph of fed and starved larvae. The straight lines marked 1 and 2 represent the increase in the sodium level in the haemolymph of fed and starved larvae, the vertical extent of these lines being equal to the difference between the initial and final flame photometer readings. Tangents to the radioactivity curves at their points of origin provide estimates for the initial influx and outflux. These estimates must be more accurate than the ones discussed above because the specific activity of the medium was measured accurately. Estimates of the initial fluxes are given in Table 1.

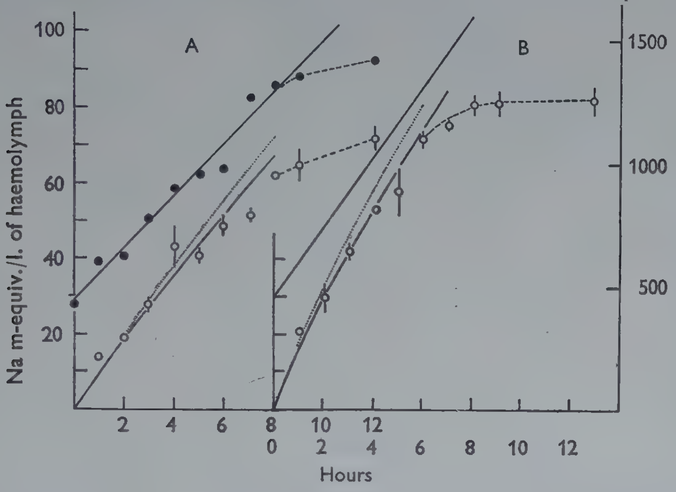

Fig. 3. A: ● = sodium level (flame photometer) in the haemolymph of starved sodium-deficient larvae—the straight line drawn through these points is the regression line for sodium level on time; ○ = influx of labelled sodium into the haemolymph of starved sodium-deficient larvae—the curve in solid line drawn through these points was calculated according to equation (1), that in dotted line according to equation (2). B: the straight line is an estimate of the net transport of sodium (see text). Other symbols as for Fig. 3 A.

Table 1

Expt. no.	State of larvae	Rate of net transport of Na (mM./l./hr.)	Initial influx of Na (mM./l./hr.)	Initial outflux of Na (mM./l./hr.)
13	Fed	39·5*	88·5	49†
14	Fed	50·5*	120†	69·5
12	Starved	8·35*	17·5	9·11†
13	Starved	8·5*	14·4	5·85†
14	Starved	9·34*	18†	8·66
19	Starved	6·96	10†	3·04

* Indicates a rough estimate from a few flame photometer samples.
† Indicates that a flux was found directly.

Fig. 4 gives information about the steady-state (or nearly steady-state) exchange which occurs after the net transport of sodium. The larvae were reared in distilled water; when they reached the 4th instar they were put into unlabelled medium from which they took up sodium. At zero time they were put into a labelled medium. The increase in labelled sodium in the haemolymph was noted, and the sodium level in the haemolymph was followed by flame photometry. In the case of starved larvae the sodium level in the haemolymph was constant so that permeability constants could be found (cf. Stobbart, 1959). For these larvae: $K_{in} = 0{\cdot}474$ hr.$^{-1}$, $T_{\frac{1}{2}} = 68$ hr., $K_{out} = 0{\cdot}0102$ hr.$^{-1}$, $[Na_{in}] = 93$ mM./l., flux of Na = 0·95 mM./l./hr. These values are all very close to the corresponding values for starved larvae reared under normal conditions (Stobbart, 1959; Treherne, 1954). In the case of fed larvae there was a slow rectilinear increase in haemolymph sodium. The rate of net transport was 0·499 mM./l./hr., S.D. = 0·0479, $P \cong 0{\cdot}0025$. The initial influx was 10·59 mM./l./hr. and the initial outflux therefore 10·09. In normal fed larvae the flux in either direction during steady-state exchange is between 7·15 and 8·84 mM./l./hr. It is reasonable to suppose that when the slow net transport is completed the fluxes would fall to this value as the flux in the starved larvae is very close to that in starved normal larvae. But it will be difficult to investigate this point as the fed larvae pupate soon after 60 hr. These results together with those in Table 1 show that the initial values of influx and outflux during net transport are much greater than the fluxes which occur during steady-state exchange.

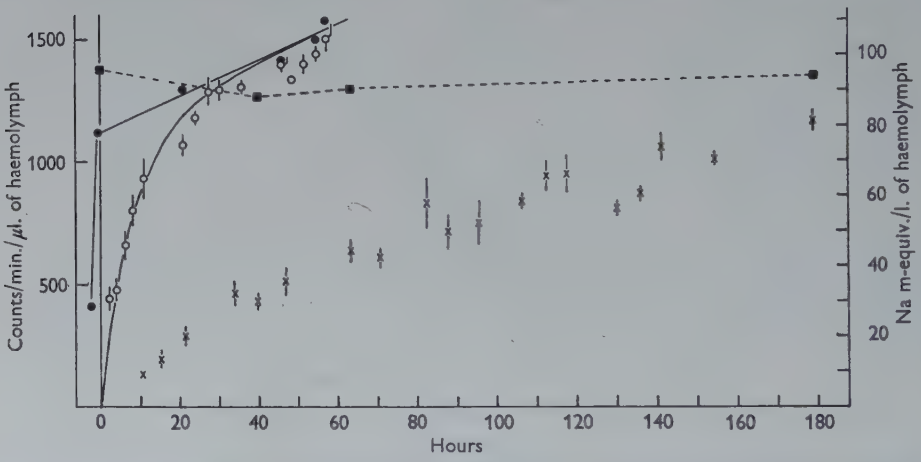

Fig. 4. ● = sodium level (flame photometer) in the haemolymph of fed larvae reared under sodium-deficient conditions and allowed to take up sodium from an unlabelled medium before entry into the labelled medium at zero time—the straight line drawn through the last five points is the regression line for sodium level on time; ■ = as above but for starved larvae; ○ = influx of labelled sodium into the haemolymph of the fed larvae—the curve drawn through the points was calculated according to equation (1), the curve calculated according to equation (2) is very similar and is not shown; × = as above but for starved larvae.

The net transport of sodium and the fluxes characteristic of it occur almost entirely through the anal papillae. Papilla-less starved sodium-deficient larvae are unable to effect a net transport inwards over a period of 14 hr. and the flux (the same in both directions) is small, about 0·2 mM./l./hr. Papilla-less fed sodium-deficient larvae are however able to effect an appreciable net transport (presumably through the gut) over the period 30–50 hr. (Fig. 5B, C). The evidence suggests that the larvae become adapted to perform this net transport after 20–40 hr. During the first hour, however, it is clear that the gut makes a negligible contribution to the net transport and its associated fluxes.

During net transport by starved sodium-deficient larvae no change in weight could be detected, nor could any change in weight be detected in starved controls kept in distilled water. The method would have detected changes greater than 10%. It therefore seems reasonable to suppose that during net transport the volume of haemolymph stays constant as any increase in weight due to gain of salts must have been negligible.

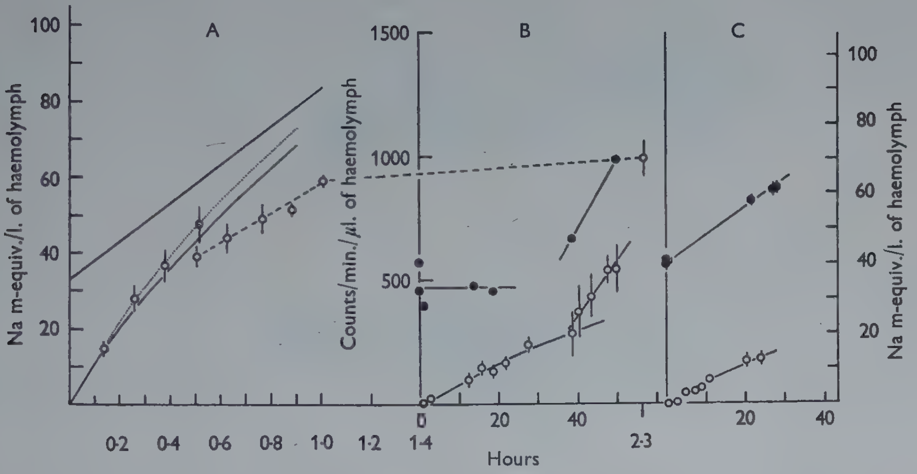

Fig. 5. A: the straight line is an estimate of the net transport of sodium by fed sodium-deficient larvae (see text); ○ = influx of labelled sodium into the haemolymph of fed sodium-deficient larvae. The curve in solid line was calculated according to equation (1), that in dotted line according to equation (2). B: ● = sodium level (flame photometer) in the haemolymph of fed sodium-deficient papilla-less larvae; ○ = influx of labelled sodium into the haemolymph of these larvae. C: symbols as for Fig. B.

It is clear that the larvae can collect sodium from very dilute media. The characteristics of steady-state exchange under sodium-deficient conditions are of interest and were studied in starved larvae reared under sodium-deficient conditions and then equilibrated in a medium diluted to give a sodium concentration of 0·003 mM./l. The exchange was followed only for 16 hr. During this time $[\mathrm{Na}_{\mathrm{in}}]$ remained steady at 57·8 mM./l. and the flux of sodium was 0·455 mM./l. in either direction. By comparison with results given earlier (Stobbart, 1959) it can be seen that when $[\mathrm{Na}_{\mathrm{out}}]$ is reduced by a factor of 0·0015, $[\mathrm{Na}_{\mathrm{in}}]$ and the fluxes are reduced by a factor of about 0·5.

Physostigmine sulphate, when applied to the outside of the larva, was found not to inhibit specifically the net transport of sodium. This is in contrast to the results of Koch (1954) obtained with other arthropod tissues.

Considerable cytological differences exist between the anal papillae of fed and of starved larvae (Stobbart, 1959). Similar differences exist between papillae of fed and of starved sodium-deficient larvae (Fig. 6). In this case the thickness of the cytoplasm of 'fed papillae' is more variable, there is no clear-cut striated region, and the cytoplasm contains many elongated bodies which are arranged so as to be roughly normal to the cuticle. The cytoplasm of 'starved papillae', however, does show a definite striated region similar to that described earlier. The nuclei of both fed and starved papillae are found inside vesicles in the cytoplasm. The probable significance of cytological differences between papillae of fed and starved larvae was discussed earlier. The papillae were fixed in Carnoy's fluid and were stained with iron haematoxylin and eosin.

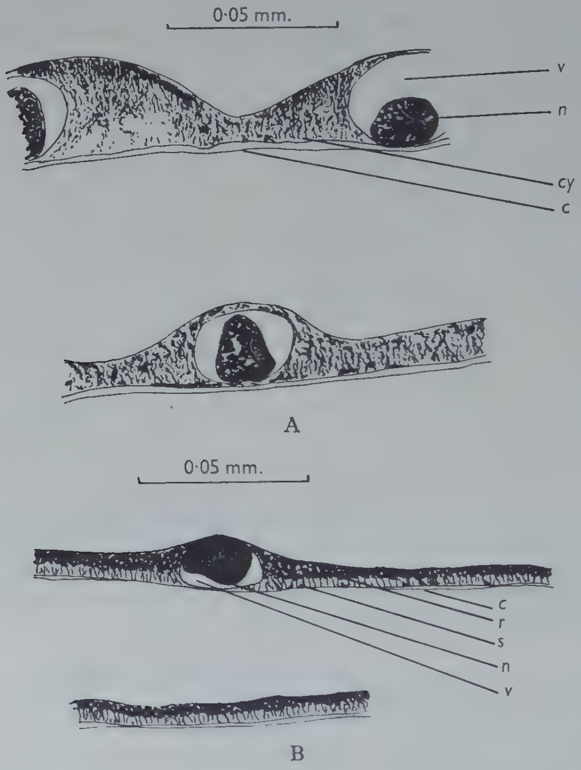

Fig. 6. Semi-diagrammatic drawings made from photomicrographs of fixed and stained anal papillae. The drawings represent longitudinal sections through the papillae. A: papillae of fed larvae; B: papillae of starved larvae. *c*, cuticle; *cy*, cytoplasm (fed larvae); *s*, striated region of cytoplasm; *r*, reticular region of cytoplasm; *v*, vesicle; *n*, nucleus.

IV. DISCUSSION

It has been clearly shown that this larva can transport sodium from the medium into the haemolymph in the face of steep concentration gradients, and previous work (Wigglesworth, 1938; Ramsay, 1953) has shown that sodium occupies a dominant position among the inorganic constituents of the haemolymph. Because of these facts, and by comparison with the situation in other tissues, I shall assume that sodium is actively pumped into the haemolymph. I shall also assume as a first approximation that the larva consists of only the haemolymph compartment (Treherne, 1954).

With the techniques used in this work a large part of the net transport of sodium from the medium to the haemolymph appears to proceed at a constant rate. Although the techniques would not have detected slight variations in rate of net transport they would certainly have detected marked ones. It seems reasonable therefore to assume that the rate of net transport is largely constant.

The rate of net transport may be increased roughly fivefold by feeding the larvae; and net transport, together with the fluxes characteristic of it, occur almost entirely through the anal papillae. The steady-state exchange of sodium may be increased roughly sixfold by feeding and it occurs principally through the anal papillae (Stobbart, 1959). These facts suggest that both net transport and exchange are brought about by some carrier mechanism in the papillae which is linked to the metabolism of the larva.

During net transport of sodium

$$m_i - m_o = k \text{ (roughly)},$$

where m_i = influx and m_o = outflux. In the case where sodium-deficient larvae are put into a labelled medium the influx is always of the labelled population in the medium while the outflux is of the initially unlabelled population in the haemolymph. If $m_o = 0$ then no exchange between the two populations will occur, and the influx of labelled sodium will be identical with the net transport of sodium. If $m_o > 0$ then some exchange will occur. Assume that $m_o > 0$ and that during the net transport the values of m_i and m_o do not alter. In this case it can be shown that

$$x = k\left[t + \omega - \frac{\omega^{(m_i/k)}}{(t+\omega)^{(m_o/k)}}\right], \tag{1}$$

where

x = concentration of labelled sodium in the haemolymph at any time

$k = m_i - m_o$

t = time.

$$\omega = \frac{S_o}{m_i - m_o},$$

where

m_i = influx of sodium (mM./l. of haemolymph/hr.),

m_o = outflux of sodium (mM./l. of haemolymph/hr.),

S_0 = concentration of sodium initially present in the haemolymph.

As t increases the term $\omega^{(m_i/k)}/[(t+\omega)^{(m_o/k)}]$ diminishes and the curve of this equation degenerates into a straight line. When m_i/k and m_o/k are large a lot of exchange occurs relative to net transport. The type of curve described by this equation is shown by curves A and B of Fig. 7.

Consider now the case where $m_i - m_o = k$, but $m_o = k_1 S$, where S = total sodium concentration in the haemolymph at any time and k_1 is an arbitrary constant. Under these conditions it can be shown that

$$x = (S_0 + kt) - S_0 e^{-k_1 t}. \tag{2}$$

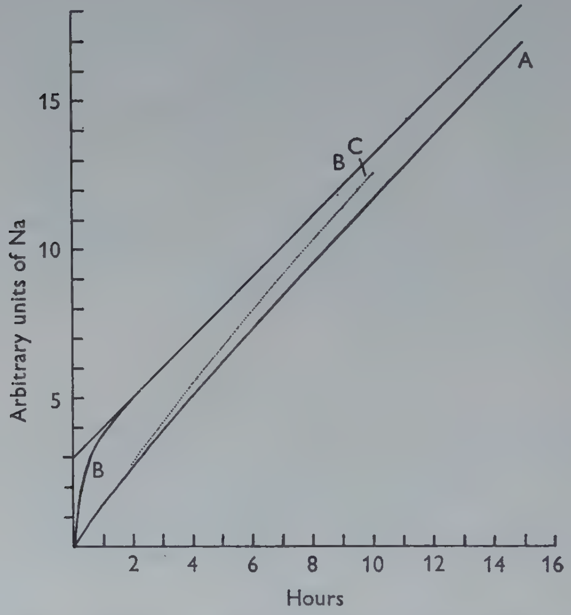

Fig. 7. Theoretical curves calculated according to equations (1) and (2) for a system bringing about a net transport of sodium of 1 unit/hr., and containing initially 3 units of sodium. It was put into a labelled medium at zero time. The straight line represents the net transport. The curves labelled A and B were calculated according to equation (1) on the basis of the following values: curve A influx = 1·5 units/hr., outflux = 0·5 unit/hr.; curve B influx = 10 units/hr., outflux = 9 units/hr.; curve C was calculated according to equation (2) on the basis of an initial influx of 1·5 units/hr., and initial outflux of 0·5 unit/hr. and a value for k_1 of 0·167. The curve calculated according to equation (2) on the basis of an initial influx of 10 units/hr., an initial outflux of 9 units/hr., and a value for k_1 of 3 approximates very closely to curve B and so it is not shown.

(In fact it should be supposed that m_o is proportional to the electrochemical activity of the sodium in the haemolymph at any time. This was not done as no information is yet available about any potential difference between haemolymph and medium during net transport.) The curve of this equation also degenerates (with increasing t) into a straight line. The curves described by (2) are similar to those described by (1). When the fluxes are of the same order of size as the net transport the uptake of labelled sodium is quicker according to (2) (Fig. 7, curve C). When the fluxes are several times larger than the net transport the curves described by (1) and (2) approximate closely, although the uptake is slightly quicker according to (2). I am indebted to Dr I. M. Glynn of the Physiological Laboratory of the University of Cambridge for the derivations of these equations (see Appendix).

Figs. 3A and B show the uptake of labelled sodium during net transport from a labelled medium by starved larvae. The curves drawn through the radioactivity points are calculated according to (1) and (2) on the basis of estimates available for the initial fluxes (Table 1). k_1 was found from the relationship

$$m'_o = k_1 S_0,$$

where m'_o is the initial outflux. The straight line in Fig. 3B is an estimate of the net transport. Its vertical extent is equal to the difference between the initial and final flame photometer readings given in Fig. 2B and the net transport is assumed to be completed in 8 hr. For the larvae of Fig. 3A the initial influx and outflux were 10 and 3·04 mM./l./hr. and k_1 was 0·105. For those of Fig. 3B the initial influx and outflux were 18 and 8·66 mM./l./hr. and k_1 was 0·292. In the case of these starved larvae the curves calculated according to (1) fit the points for uptake of radioactivity well and slightly better than those calculated according to (2), but the significance of this difference in fit is probably doubtful. The uptake of labelled sodium by fed larvae during the slow phase of net transport is shown in Fig. 4. The curve drawn through the radioactivity points was calculated according to (1); the values of influx and outflux were 10·59 and 10·09 mM./l./hr. The corresponding curve calculated according to (2) is very similar and is not shown. The curves fit the points well. The uptake of labelled sodium by fed larvae during the rapid phase of net transport is shown in Fig. 5A. The straight line is an estimate of net transport similar to the one given in Fig. 3B. Curves calculated according to (1) and (2) are drawn through the radioactivity points. The values of influx and outflux were 120 and 69·5 mM./l./hr. and that of k_1 was 2·1. Over roughly the first half hour both curves fit the points well, but after this time the uptake of radioactivity is slower than that predicted, and it appears to follow a straight line roughly parallel to the estimated net transport. This result obviously needs to be confirmed. If it is true then it follows that outflux is abolished and influx reduced during the latter half of the net transport.

It appears from the foregoing considerations that there is no vast increase in the fluxes during net transport in the case of starved larvae and in the case of fed larvae during the slow phase of net transport. During the rapid phase of net transport by fed larvae, however, there may be a reduction in the fluxes. It should prove possible to obtain more information about the net transport by measuring the fluxes directly at different times during the net transport.

The results which have been presented here are compatible with the sort of sodium pump which was suggested earlier to account for the steady-state exchange (Stobbart, 1959). The pump is supposed to work in conjunction with something similar to an exchange diffusion mechanism (E.D.M.) which is confined to an osmotic barrier in the anal papillae. The pump is supposed to split off from the E.D.M. at the inner surface of the barrier sufficient sodium to balance any passive losses or to bring the sodium level in the haemolymph back to the normal value. The sodium attached to the E.D.M. is free to exchange with that in the haemolymph or medium. An increase in the fluxes could be brought about by (1) an increased rate of movement of E.D.M.'s, (ii) an increase in the amount of sodium carried by each E.D.M., (iii) a synthesis of more E.D.M.'s, (iv) an increase in any possible enzymic catalysis of exchange of sodium between E.D.M. and medium of haemolymph. Both the exchange and the pumping of sodium are increased by feeding in sodium-deficient larvae. When sodium-deficient larvae are put into the medium, net transport and the large fluxes associated with it are observed straight away. This suggests (in terms of the proposed sodium pump) that in sodium-deficient larvae in a sodium-deficient medium the capacity of the E.D.M. to carry sodium is increased in an attempt to present the pump with sodium originating from the medium outside. If the E.D.M. has a high affinity for sodium (as is the case in *Aëdes*) it might act as a mechanism for moving sodium through an osmotic barrier and presenting it to a pump. Sodium not removed by the pump would move back and forth across the barrier and could give rise to an exchange diffusion component in the fluxes (cf. Mitchell & Moyle, 1953). A mechanism of this sort should not cause an outflux of sodium from the larva when sodium is missing from the medium outside. Seen in this light, exchange diffusion is not necessarily a useless by-product of metabolism. The increase in steady-state exchange brought about by feeding (Stobbart, 1959) may mean that the pump is in a greater state of readiness to deal with emergencies as the influx of sodium is roughly 6 times greater than it is in starved larvae. Exchange diffusions that are apparently useless do occur (Croghan, 1958*a*, *b*). Perhaps exchange diffusion is a general characteristic of biological membranes and is one which in certain cases lends itself to the development of ionic pumps.

It is possible that the increased outflux during net transport is a passive process caused by an increased electrochemical activity of sodium in the haemolymph due to the active influx. Until information about the electrochemical activity is available it is not possible to say whether the increased outflux is more likely to be due to passive diffusion or to be mediated by some sort of carrier.

The net transport of sodium slows down, or slows down and stops, quite abruptly when the level of sodium in the haemolymph approaches that of normal larvae. This rapid control would appear to be worth investigation. In fed sodium-deficient larvae the rate of net transport slows down abruptly, but does not stop, when the sodium level reaches 80–90 mM./l. The reasons for this are obscure. A slow net transport cannot be detected in fed normal larvae. Perhaps when the sodium level reaches a certain critical value most of the energy which would have been used for net transport is diverted to other ends.

V. SUMMARY

1. The net transport of sodium into the haemolymph by sodium-deficient 4th-instar larvae of *Aëdes aegypti* (L.) has been studied by means of flame photometry. The fluxes associated with this net transport have been studied by means of ^{22}Na.

2. The net transport is much more rapid in fed larvae (rate about 50 mM./l./hr.)

than in starved larvae (rate about 10 mM./l./hr.). The fluxes are also much greater in the fed larvae.

3. The fluxes associated with net transport in fed and starved larvae are much greater (initially at any rate) than the fluxes occurring in normal fed and starved larvae during steady-state exchange.

4. In both fed and starved larvae almost all the net transport and the fluxes associated with it occur through the anal papillae, so these organs must be responsible for almost all the difference between fed and starved larvae in the rate of net transport and fluxes.

5. Cytological changes in the anal papillae following upon feeding and starvation are described.

6. The results are discussed in terms of possible carrier mechanisms in the anal papillae.

I should like to thank Dr J. A. Kitching, F.R.S., under whose supervision this work was done, for guidance and encouragement, and Prof. J. E. Harris, F.R.S., for his active interest in the problem. I am grateful to Dr I. M. Glynn for providing the derivations of equations (1) and (2), to Dr K. Zerahn for some helpful discussion, and to Dr K. M. Smith, F.R.S., for letting me use the photographic facilities of the A.R.C. Virus Research Unit, Cambridge.

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APPENDIX

Derivation of equation (1)

During the rectilinear net transport let

$$\text{influx (assumed constant)} = m_i,$$
$$\text{outflux (assumed constant)} = m_o,$$

In addition let:

t = time,

amount of Na originally present inside be S_0,

amount of Na present inside at any time be S,

amount of Na* present inside at any time be x.

The medium outside is assumed to stay constant

Then
$$\frac{dx}{dt} = m_i - m_o r, \tag{i}$$

when $r = x/S$ = relative specific activity inside at any time (taking the outside medium as 1),

and
$$\frac{ds}{dt} = m_i - m_o, \tag{ii}$$

therefore
$$S = (m_i - m_o)t + S_0. \tag{iii}$$

Therefore
$$\frac{dx}{dt} + \frac{m_o x}{(m_i - m_o)t + S_0} = m_i. \tag{iv}$$

Write
$$P = \frac{m_o}{(m_i - m_o)t + S_0}$$

Multiplying both sides of (iv) by $e^{\int P dt}$ we get

$$\frac{dx}{dt} e^{\int P dt} + Px\, e^{\int P dt} = m_i e^{\int P dt}, \tag{v}$$

therefore
$$\frac{d}{dt}\left(x e^{\int P dt}\right) = m_i e^{\int P dt}, \tag{vi}$$

$$x e^{\int P dt} = \int m_i\, e^{\int P dt}\, dt. \tag{vii}$$

Now P by definition $= \dfrac{m_o}{(m_i - m_o)t + S_0}$,

therefore
$$e^{\int P dt} = \exp\left[\int \frac{m_o}{(m_i - m_o)t + S_0}\, dt\right], \tag{viii}$$

$$= \exp\left[\frac{m_o}{m_i - m_o} \log_e\left(t + \frac{S_0}{m_i - m_o}\right)\right] \tag{ix}$$

$$= \left(t + \frac{S_0}{m_i - m_o}\right)^{m_o/(m_i - m_o)} \tag{x}$$

Substituting in (vii)

$$x\left(t+\frac{S_0}{m_i-m_o}\right)^{m_o/(m_i-m_o)} = m_i\int\left(t+\frac{S_0}{m_i-m_o}\right)^{m_o/(m_i-{}_om)} dt$$

$$= (m_i-m_o)\left(t+\frac{S_0}{m_i-m_o}\right)^{m_i/(m_i-m_o)}+c. \tag{xii}$$

To obtain c put x = 0 when t = 0 since there is no radioactivity inside at the start of the experiment

$$C = -(m_i-m_o)\left(\frac{S_0}{m_i-m_o}\right)^{m_i/(m_i-m_o)} \tag{xiii}$$

From (xii)

$$x\left(t+\frac{S_0}{m_i-m_o}\right)^{m_o/(m_i-m_o)} = (m_i-m_o)\left[\left(t+\frac{S_0}{m_i-m_o}\right)^{m_i/(m_i-m_o)}-\left(\frac{S_0}{m_i-m_o}\right)^{m_i/(m_i-m_o)}\right], \tag{xiv}$$

therefore

$$x = (m_i-m_o)\left[\left(t+\frac{S_0}{m_i-m_o}\right)-\frac{\left(\frac{S_0}{m_i-m_o}\right)^{m_i/(m_i-m_o)}}{\left(t+\frac{S_0}{m_i-m_o}\right)^{m_o/(m_i-m_o)}}\right] \tag{xv}$$

This equation gives x explicitly as a function of t. Call (m_i-m_o) k and $\left(\frac{S_0}{m_i-m_o}\right)$ ω, then we have

$$x = k\left[t+\omega-\frac{\omega^{m_i/k}}{(t+\omega^{m_o/k}}\right], \tag{xvi}$$

Derivation of equation (2)

During the rectilinear net transport:

let m_i be the influx at any time;

let m_o be the outflux at any time;

let $m_i-m_o = k$, where k is a constant.

In addition:

let $m_o = k_1 S$ where k_1 is a constant,

and let S_0, S, x, and t be defined as before.

The medium outside is assumed to stay constant. Then

$$\frac{dx}{dt} = m_i-m_o\,r, \tag{i}$$

when $r = x/S$ = relative specific activity inside at any time (taking the outside medium as 1).

$$\frac{dx}{dt} = (k+k_1S)-\frac{x}{S}k_1S, \tag{ii}$$

$$= k+k_1S-x\,k_1, \tag{iii}$$

Since $S = kt + S_0$

$$\frac{dx}{dt} = k + k_1(kt + S_0) - xk_1, \tag{iv}$$

$$= k + k_1 S_0 + k_1 kt - xk_1, \tag{v}$$

$$= a + bt - cx, \tag{vi}$$

where $a = k + k_1 S_0$, $b = k_1 k$, $c = k_1$; therefore

$$\frac{dx}{dt} + cx = a + bt. \tag{vii}$$

Multiplying both sides of (vii) by e^{ct} we get

$$e^{ct}\frac{dx}{dt} + e^{ct}\,cx = e^{ct}(a + bt) \tag{viii}$$

$$\text{L.H.S.} = \frac{d}{dt}(e^{ct}x)$$

Integrating both sides with respect to t

$$e^{ct}x = \int e^{ct}(a + bt)dt, \tag{ix}$$

$$= \int a e^{ct}\,dt + \int bt e^{ct}\,dt, \tag{x}$$

$$= \frac{a}{c}e^{ct} + b\int t e^{ct}\,dt, \tag{xi}$$

$$= \frac{a}{c}e^{ct} + \frac{bt}{c}e^{ct} - \frac{b}{c^2}e^{ct} + \text{constant}, \tag{xii}$$

$$= e^{ct}\left(\frac{ac + cbt - b}{c^2}\right) + \text{constant}. \tag{xiii}$$

Substituting for a, b and c and simplifying

$$e^{k_1 t}x = e^{k_1 t}(S_0 + kt) + \text{constant}. \tag{xiv}$$

Dividing both sides by $e^{k_1 t}$

$$x = (S_0 + kt) + \text{constant}\,.\,e^{k_1 t} \tag{xv}$$

When $t = 0$, $x = 0$, therefore

$$\text{constant} = -S_0,$$

therefore

$$x = (S_0 + kt) - S_0 e^{-k_1 t}. \tag{xvi}$$

LOCOMOTOR MOVEMENTS IN THE SPINAL PIGEON

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(*Received* 15 *March* 1960)

(With Plate 14)

INTRODUCTION

It has long been known that after isolation from the rest of the central nervous system the spinal cord of vertebrates is, under favourable circumstances, still capable of showing reflex activity. In the spinal bird reflexes affecting the lower part of the body are sometimes very much in evidence. This was pointed out by Singer (1884), Tarchanoff (1895), Trendelenburg (1906), Baglioni & Matteucci (1910). Nearly all the experiments have been carried out on pigeons and ducks. Singer was the first to describe, among the different reflexes, the alternating flexor and extensor reflexes of the hind limbs in spinal pigeons. This finding was confirmed by all other investigators.

Although the alternating movements of the hind limbs, which have also been described in spinal mammals (dog), constitute an essential element in the completion of the movements of walking, no true locomotor movements of the hind limbs have been, to my knowledge, elicited in the spinal bird. Legalois (1812) and later Tarchanoff (1884) did describe a kind of walking movement in decapitated birds. But this was only observed directly after decapitation and was of very short duration. It is important to note that these walking movements of the legs were accompanied by violent wingbeats. These phenomena, which are caused by strong excitation of the spinal cord, certainly do not have the character of normal walking movements. As Flourens (1824) and all the other investigators after him have rightly shown, true locomotion is impossible in birds after transection of the spinal cord.

In the author's experiments on dogs (1939), in which the lumbosacral part of the spinal cord was isolated from the rest of the central nervous system by transection, it could be observed that a spinal dog could execute alternating walking movements with the hind limbs, when by means of strong contractions of the dorsal muscles the lower part of the body is drawn up till the hind legs touch the ground with the soles of the feet. The contact of the soles with the ground causes the extensor tone in the hind legs to increase, and reflexly alternating movements may appear when the fore-part of the animal moves by means of the forelegs.

METHOD

It seemed probable that locomotor movements of the hind limbs would also occur in birds, if they were placed in such a position that they could touch the ground with extended legs. For this purpose a little four-wheeled carriage was constructed, on which a pigeon could be fixed in a normal position and in such a way that with its legs extended it could reach the ground. The height of the carriage could be adjusted.

The transection of the spinal cord was executed under ether anaesthesia and the section was made just cranially to the intumescentia lumbosacralis. Directly after the transection the pigeons could not stand, still less walk. After the effect of the ether had passed off, all reflexes of legs and tail, formerly described by the author and other investigators, could easily be elicited by stimulation of the legs and the coccyx. The alternating movements of the hind limbs could also be elicited by stimulation not only of the legs but also of the skin of the coccyx.

RESULTS

The tone of the muscles of the hind limbs in the spinal pigeon was very low during the first few days and recovered very gradually. Only after about 2 weeks could a distinct increase of tone be observed. But for the rest of its life the pigeon rested on its belly and was unable to stand or walk. When startled it moved by means of powerful wingbeats. In the beginning the legs remained extended and motionless, or drawn up against the body. After about 7 days movements of the legs could be observed during the wingbeats; the legs were held extended backwards. Later we also observed the alternating backward extensions of the legs.

When a spinal pigeon was fixed on the above-described carriage in a normal upright posture and in such a way that the two legs found support on the ground with soles and toes, it was possible to elicit, by stimulation of a leg or of the coccyx, alternating movements of the two hind limbs that looked exactly like real walking movements. When walking the pigeon placed its legs on the ground with the toes extended as does a normal pigeon. In this way a spinal pigeon can cover relatively great distances. Being bound down to the carriage, the wings cannot take part in these movements. The rhythm of the alternating walking movements of spinal pigeon could be considerably speeded up when the anus was stimulated with a probe. Naturally the spinal pigeon could not avoid obstacles in its path.

These experiments have shown that 2 or 3 months after the transection a spinal pigeon can execute walking movements when it is placed in such a position that it touches the ground with the soles of the feet and the toes.

When, during an experiment, a pigeon freed itself from the carriage it moved by means of wingbeats. As the wings had been clipped it could not fly up but moved along the ground executing movements with the wings and with the legs simultaneously. The movements of the legs were alternating, but not co-ordinated with the wingbeats.

The fact that the spinal pigeon, placed upright on a carriage where it can stand on its feet and will find support on the ground, is capable of true locomotor movements, indicates that the locomotor movements of the legs in a bird can be co-ordinated independently by the isolated lumbosacral cord. If no locomotor movements of the legs are observed under normal circumstances in the spinal bird, this is because the higher centres of the nervous system, which ensure the normal position of the bird, have been eliminated. After isolation of the lumbosacral cord a bird standing on its legs cannot keep its body in the normal upright posture—it rests on its belly and only moves by means of wingbeats.

Although the tone of the muscles of the hind limbs increases progressively the extensor tone remains insufficient for the support of the body in an upright posture, even when the forepart of the body is brought into a normal posture and supported by the hand.

In my experiments with spinal dogs I was able to establish that with these animals a considerable increase of the extensor tone of the hind limbs could be elicited by pressure on the soles of the feet, so that they could more or less support the lower part of the body. In the spinal pigeon, however, I have never succeeded in obtaining a considerable increase of extensor tone in the legs by pressing on the soles or by spreading the toes.

I shall now describe some other experiments which also indicate a notable functional autonomy of the isolated spinal cord in birds.

About 6 weeks after transection of the cord of the pigeon I was able repeatedly to elicit an acceleration of the walking movements with simultaneously rhythmical spreading of the tail-feathers by stimulation of the anus (or the anal region) with a probe. Similar reactions in the lower part of the body could be elicited by painful stimuli applied to the skin in the coccyx area.

Stimulation by pinching the more forward skin areas, which lay close beneath the section through the spinal cord, caused a backward movement, the pigeon taking some alternating steps backwards with the hind limbs.

When the stimuli applied were too strong, violent irregular movements of the legs occurred; the tail feathers were spread and moved up or down. As a result of these violent movements the forepart of the pigeon was often involved in this reaction; the animal usually freed itself or upset the carriage. All these movements can be explained as flight and evasion reflexes, which can be brought about by the isolated lumbosacral spinal cord.

The above observations prove that the isolated lumbosacral spinal cord can co-ordinate various movements of the legs and of the tail, according to the strength and location of the stimulus applied.

From the above experiments it appears that walking movements in spinal pigeons can also be elicited by peripheral stimulation of the hindpart of the body. By peripheral stimuli which arise in the muscles, joints and skin during the movement of the legs, the activity of the locomotor centres in the lumbosacral cord can be maintained for a considerable time. All these facts indicate a high degree of autonomy of the locomotor centres in the spinal cord.

While in normal pigeons the impulses that give rise to locomotion by walking are mainly transmitted from the brain to the lumbosacral cord, the spinal pigeon depends entirely on afferent impulses from the periphery to the isolated spinal cord.

When a spinal pigeon is artificially brought into a normal upright posture by fixing it on the carriage described above with the legs finding support on the ground, peripheral stimulation can put the locomotor centres in the lumbosacral spinal cord into action. The alternating flexor and extensor reflexes of the legs can maintain the activity of the centres in the isolated spinal cord during a relatively long period. Similar observations were made on the spinal dog.

Thus the walking of birds is a more or less automatic process which is governed by the lumbosacral part of the spinal cord. The locomotor centres, however, are under the continuous influence of the higher centres, by which locomotion is normally initiated. Besides these, the impulses generated by movements in the hind limbs and conducted to the spinal cord are also of importance. After transection of the spinal cord the rhythmical function of the locomotor centres in the lumbosacral spinal cord can be maintained and regulated by the movements of the legs. In this connexion it should be noted that the isolated lumbosacral spinal cord in the bird is eminently capable of bringing about different movements of legs and tail in response to different stimuli acting on the hindpart of the body.

CONCLUSIONS

1. The lumbosacral spinal cord is able to co-ordinate locomotor movements of the hind limbs.
2. A spinal bird under normal circumstances is unable to walk since it can no longer maintain the upright posture in consequence of the disconnexion of the higher centres.
3. When a spinal pigeon is fixed in a carriage, so that its legs reach the ground, true walking movements in the the hind limbs can be elicited by painful stimuli applied to the hindpart of the body. In this way the pigeon can move over relatively great distances.
4. A flight or evasion reflex can also be elicited by stimulation of the skin of the coccyx and anus.
5. The spinal cord of birds shows a high degree of autonomy of the lower centres in the co-ordination of walking movements. The higher centres initiate and regulate these movements.

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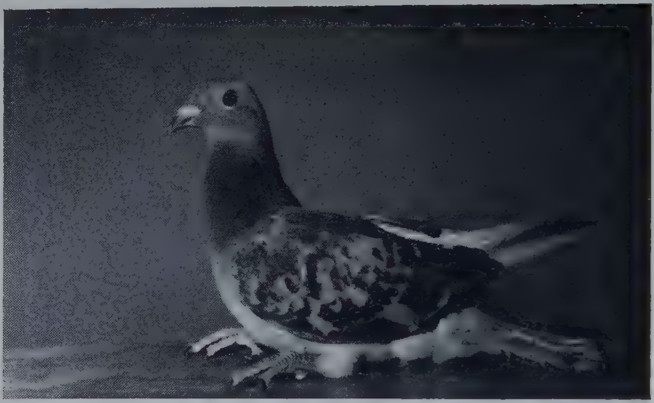

Fig. 1

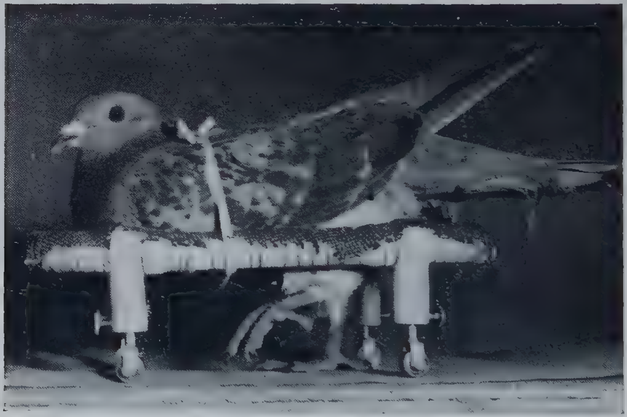

Fig. 2

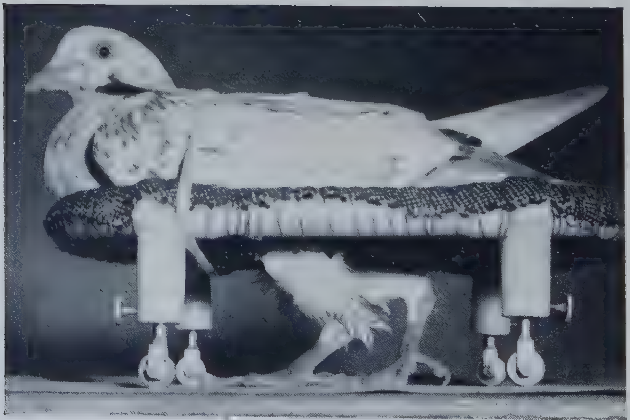

Fig. 3

TEN CATE—LOCOMOTOR MOVEMENTS IN THE SPINAL PIGEON

(*Facing p.* 613)

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EXPLANATION OF PLATE

Fig. 1. Spinal pigeon resting on belly, 3 weeks after transection.

Fig. 2. Walking movements of the spinal pigeon fixed to the carriage, 2 weeks after transection.

Fig. 3. Walking movements of the spinal pigeon fixed to the carriage, 3 months after transection

SOME EFFECTS OF TEMPERATURE AND CONCENTRATION OF THE MEDIUM ON THE IONIC REGULATION OF THE ISOPOD *ASELLUS AQUATICUS* (L.)

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INTRODUCTION

The temperature of small bodies of fresh water may undergo considerable changes within a short period. Such changes in temperature are likely to affect the rates of all biological processes including the mechanisms responsible for the active uptake of ions from the medium and the production of a hypotonic urine. Although the osmotic uptake of water and any passive loss of ions from the body may have high temperature coefficients it is unlikely that these will be the same as the temperature coefficients of the active mechanisms. Since ions have to be replaced at the same rate as they are lost from the body if the haemolymph concentration is to be maintained at a constant level, it is of interest to investigate the means by which fresh-water poikilotherms are able to withstand the potential hazards of change in the temperature of their medium.

Wikgren (1953) has discussed the previous work in this field in relation to his own findings on *Potamobius*, *Petromyzon* and various teleosts. He has shown that the ionic regulation of *Petromyzon* is affected in two ways by a reduction in the temperature of its medium to 1–2° C: first, there is a decrease in the urine volume. This is accompanied by a decrease in salt readsorption so that the Cl concentration of the urine remains similar to that at higher temperatures. Second, the rate of ion uptake from the medium is markedly reduced. As a result there is an initial net loss of ions from the body at low temperatures.

Evidence suggesting the presence of an effective and sensitive process for the active uptake of sodium by *Asellus* has already been described (Lockwood, 1959*a*). The present report is mainly concerned with investigation into the effect of different temperatures and different concentrations of the medium on the rate of loss of ions from the body, on the ionic concentration of the haemolymph and on the rate of uptake of ions from the medium. Some data are also given of the sodium fluxes.

DEFINITIONS AND THEORETICAL CONSIDERATIONS

In this paper the term sodium 'flux' is used to indicate the total rate of sodium movement as determined using labelled sodium. 'Influx' incorporates both active transport of sodium into the animal and also exchange diffusion. Conversely, 'efflux' is made up of loss of sodium from the body by diffusion and in the urine plus exchange diffusion. Since, by definition, the component of exchange diffusion is the same in both directions at any one time, the sodium content of the body of the animal remains constant only when the active uptake of sodium balances the diffusion and urine loss.

The term 'fully loaded animal' is applied to one which has been allowed to exchange its sodium with a medium containing tracer for a time sufficient to ensure that the specific activity is the same in both animal and medium.

The following symbols have been used in this paper:

O.P._{i}	haemolymph osmotic pressure (expressed as mM/l. NaCl)
Na_{i}	haemolymph sodium concentration
Cl_{i}	haemolymph chloride concentration
Na_{o}	sodium concentration of the medium

If in the study of the exchange of sodium between an animal and its medium it is permissible to regard the internal sodium as present in a single phase, the flux may be calculated from the equation

$$R = \frac{\text{Na}_{\text{i}}}{t} \ln \left(\frac{{}^{*}\text{Na}_{\infty}}{{}^{*}\text{Na}_{\infty} - {}^{*}\text{Na}_{t}} \right) \qquad (1)$$

where R is the flux in m-equiv./l. haemolymph/hr.,

Na_{i}	is the sodium concentration of the haemolymph in the steady state,
$^{*}\text{Na}_{t}$	is the tracer activity in a sample of haemolymph at time t, and
$^{*}\text{Na}_{\infty}$	is the tracer activity in a similar sample of haemolymph when exchange of tracer with the medium is complete.

Replacing Na_{i} by 100 gives the flux in terms of % haemolymph sodium exchanged per hour.

The use of the above equation, which treats the whole sodium content of the animal as being in a single phase, is justified if the exchange of sodium between the haemolymph and any other compartment is either very rapid or very slow compared with the exchange between the haemolymph and medium. It will be shown that the exchange of sodium between haemolymph and non-haemolymph compartments in *Asellus* is slow compared with the exchange between haemolymph and medium. The above equation can therefore be used to calculate the sodium flux between the haemolymph and medium provided that its use is restricted to the first few hours of an experiment. During this time exchange between haemolymph and tissues will be small.

Prior to some of the experiments to be described in this paper each animal was fully loaded in a solution containing ^{22}Na. Depletion of the sodium content of the animal was brought about by exposing it to a current of deionized water, and the sodium-depleted animal was allowed to replace the lost sodium from the original loading solution. Since no exchange diffusion is possible if the medium contains no sodium and since there is no net effect of exchange diffusion when the specific activity of ^{22}Na is the same in the medium and in the animal, it follows that in these circumstances any change in the count rate of the animal represents a net change in its sodium content.

MATERIALS AND METHODS

Asellus aquaticus was obtained from Coe Fen, Cambridge, and in later experiments from a number of fresh-water sites around Edinburgh.

Osmotic pressures were measured by the method of Ramsay & Brown (1955), sodium by flame photometry and chloride by the first method of Ramsay, Brown & Croghan (1955). Details of the procedure and the method of haemolymph collection have already been described (Lockwood, 1959*a*). Haemolymph samples for the measurement of isotope activity were collected and stored below liquid paraffin as in the case of samples for osmotic pressure determinations. Aliquots of haemolymph were pipetted on to planchettes or polythene disks and were evaporated to dryness before counting. Sufficient haemolymph could be obtained from a single large animal to allow duplicate samples to be taken. Samples of medium were pipetted directly on to planchettes, evaporated and counted. Whole animals were counted in one of the Perspex washing-out trays previously described (Lockwood, 1959*b*).

In most cases sufficient counts were recorded to give a statistical accuracy of ±2%. The usual corrections for background and count rate were applied to the raw data and in addition, during long-term experiments, a ^{22}Na standard was counted before and after each ^{22}Na sample in order that corrections might be made for variations in the counting efficiency of the apparatus.

Animals were not fed during the course of experiments.

RESULTS

The sodium flux

Two methods have been used in the study of the sodium flux . In the first, animals were transferred from a medium to which they had been acclimatized (1·1 mM./l. NaCl) to a measured weight of a similar medium containing ^{22}Na. Aliquots of the medium were taken at intervals, dried down on to polythene disks and counted. After counting, each disk was rinsed in the medium to remove the sample and the medium was brought up to its initial weight by the addition of deionized water. Aliquots of the medium were counted several times a day for 6 days and then again after 30 days to obtain the value after complete exchange. The temperature was 20 ± 1·5° C. (Fig. 1*a*).

A semi-logarithmic plot of $M_t - M_\infty$ (where M_t is medium count at time t and M_∞ is the steady-state count) against time would be linear if a single rate constant were governing the sodium exchange. Fig. 1*b* indicates that this is not the case in *Asellus*. Two rate constants have been derived graphically from the results of this plot, the slower giving a half-time of exchange of 114 hr. and the more rapid one of 19 hr. Direct extrapolation to obtain the proportion of the total sodium

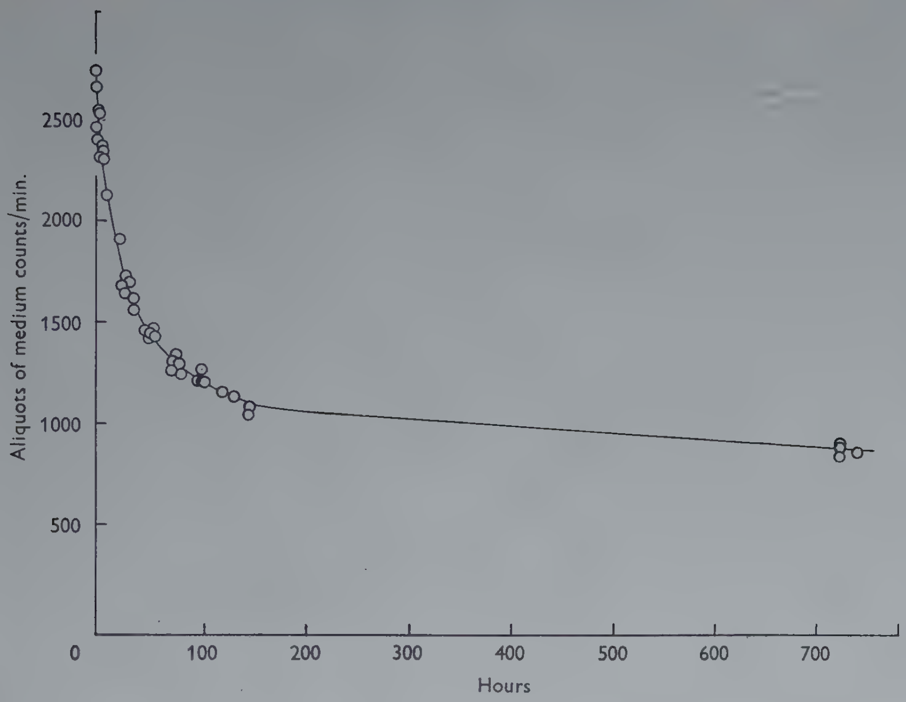

Fig. 1 *a*. Falling count of aliquots of medium during uptake and exchange of ^{22}Na by five animals.

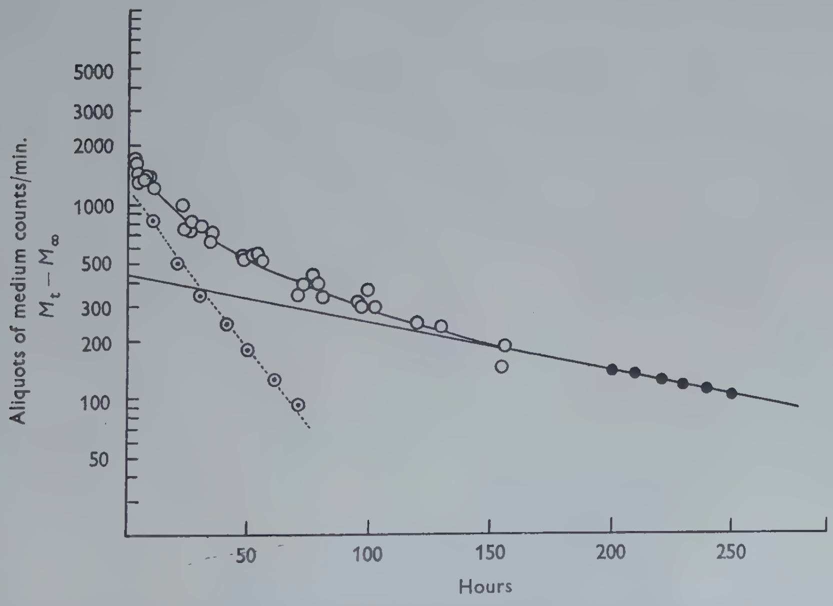

Fig. 1 *b*. Semi-logarithmic plot of data from Fig. 1 *a* to show the two compartments of Na aliquot counts ($M_t - M_\infty$) where M_t is the observed count and M_∞ the count when the exchange of ^{22}Na is complete. ——, back extrapolation for slow component of flux. · · · ·, fast component of flux.

accounted for by the slow rate is liable to error, but the value obtained of between 20 and 30% of the total sodium is similar to that found for the proportion of extrahaemolymph sodium in *Asellus* (Lockwood, 1959*c*) and it is presumed that the slow exchange corresponds to the exchange of the sodium in Zenker's organ.

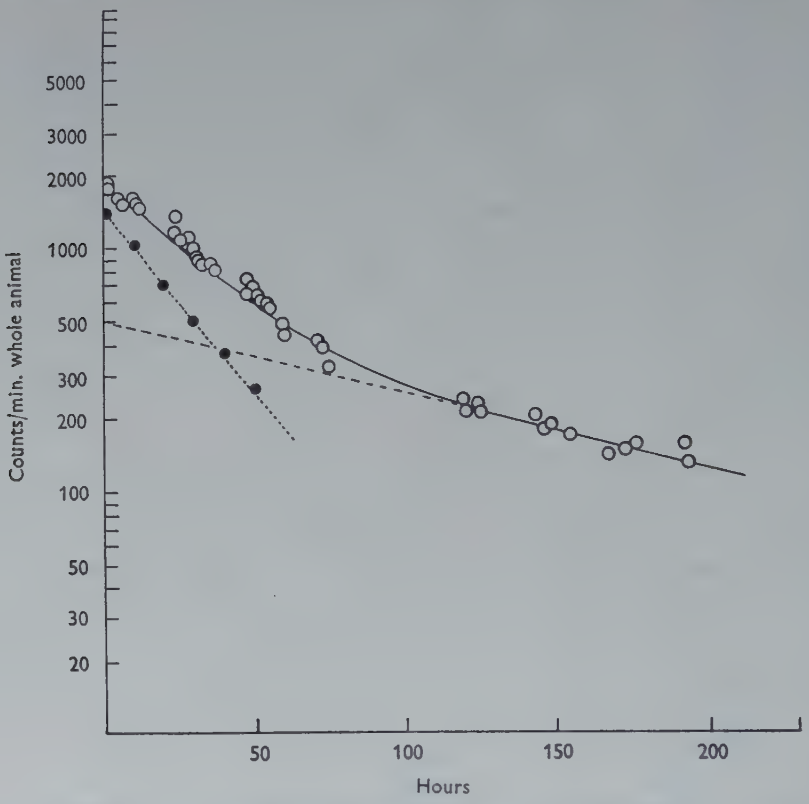

Fig. 2. Loss of ^{22}Na by a fully loaded animal washed with a non-tracer medium. ○, counts of whole animal: – – –, back extrapolation of slow component of flux: ●, fast component of flux.

In the second method the sodium flux was examined by following the decrease of activity of a fully loaded animal exposed to a current of tracer-free medium. The loss of tracer was again defined by two rate constants (Fig. 2), the half-times for exchange being 20 and 103 hr. respectively.

In these experiments, and in others involving exchange of sodium, care was taken to select animals of closely similar size since preliminary experiments had shown the existence of a relationship between the size of animal and the rate of exchange of sodium per unit weight (Fig. 3).

The effects of temperature on ion regulation

(*a*) *The response of haemolymph concentration to temperature*

Small groups of animals were kept in 1·7 mM./l. NaCl at different temperatures. After 2 weeks the haemolymph was sampled and the O.P.$_i$ and Na$_i$ of the haemolymph were compared in animals from different temperatures. The results of three similar experiments (Table 1) provide a general confirmation for the finding of Heuts (1943) that the O.P.$_i$ and Cl$_i$ of *Asellus* decrease with temperature to a minimum value at about 4° C. and then rise again at temperatures below this. The rise in O.P.$_i$ and Na$_i$ below 5° C. is not associated with an increase in the total sodium content of the body. Animals fully loaded at room temperature and then transferred to a temperature of 1° C. in the same loading medium all showed a net loss of sodium (Table 2). When the temperature was raised once more the animals showed a net uptake of sodium from the medium.

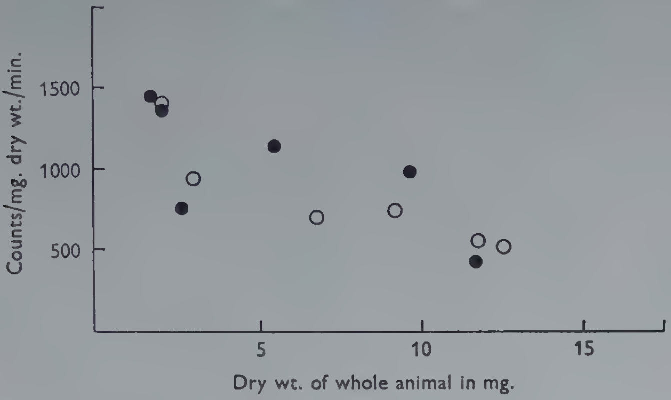

Fig. 3. Rates of exchange of sodium per unit body weight of animal. ●, ○, results derived from two similar experiments.

Table 1. *The effect of temperature difference on* O.P.$_i$ *and* Na$_i$

Experiment 1

Temperature (° C.)	O.P.$_i$ mM./l. NaCl	Na$_i$ mM./l.
1	156	—
1	165	152
1	164	145
11	142	126
11	137	126
11	142	128
24	150	137
24	142	135

Experiment 2
Conc. of medium
2·4 mM./l. NaCl

	3·5° C.	12° C.	18° C.	22° C.
Na$_i$ mM./l.	127 ± 2·7 $n = 11$	123 ± 1·8 $n = 11$	126 ± 0·9 $n = 10$	129 ± 1·4 $n = 10$

Experiment 3
Conc. of medium
57 μM./l. NaCl

	5° C.	8° C.	15° C.	20° C.	24·5° C.
Na$_i$ mM./l.	110 ± 2·6 $n = 11$	96 ± 4·2 $n = 3$	103 ± 2·5 $n = 10$	106 and 109 $n = 2$	110 ± 1·5 $n = 10$

Mean ± standard error. n = number of observations.

It is possible that the rise in the ion concentration of the haemolymph at low temperatures results from the accumulation of metabolites in the cells and a consequent water shift to the cells. A similar water shift to the cells has been postulated to explain the maintenance of a relatively high haemolymph concentration when *Asellus* is washed with deionized water (Lockwood, 1959*a*).

Animals kept in a very dilute medium (57 μM./l. NaCl) at different temperatures showed a relationship between Na$_i$ and temperature (Fig. 4) similar to that found at the higher concentration of NaCl. At all temperatures however Na$_i$ was considerably below the 'normal' level.

Table 2. *The loss of sodium from animals fully loaded at room temperature and transferred to 1° C in the same medium*

	% initial sodium remaining in body. Period at 1° C.			
Animal	1 day	2 days	3 days	4 days
1	94	—	—	—
2	92	90	84	—
3	—	95	96	—
4	—	91	87	—
5	—	98	80	71
6	—	—	—	79

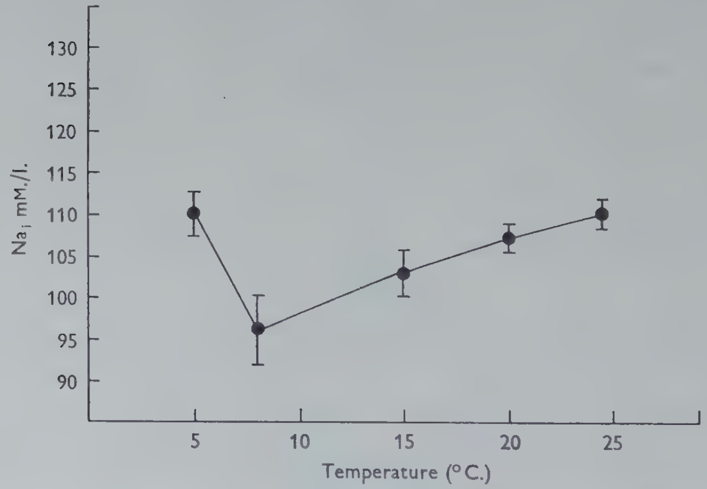

Fig. 4. The influence of temperature on the steady state Na$_i$ of animals in very dilute NaCl (57 μM./l.). ●, means: I, range of standard error.

The experimental finding on the short-term relationship between temperature and Na$_i$ and O.P.$_i$ may be compared with the observed changes in the O.P.$_i$ of *Asellus* during the year. In Fig. 5 are shown the means and standard errors for O.P.$_i$ of freshly caught individuals in different months. There is a small rise in the O.P.$_i$ as the water temperature falls in winter. During the remainder of the year, however, the O.P.$_i$ is remarkably constant and the differences from month to month are not significant.

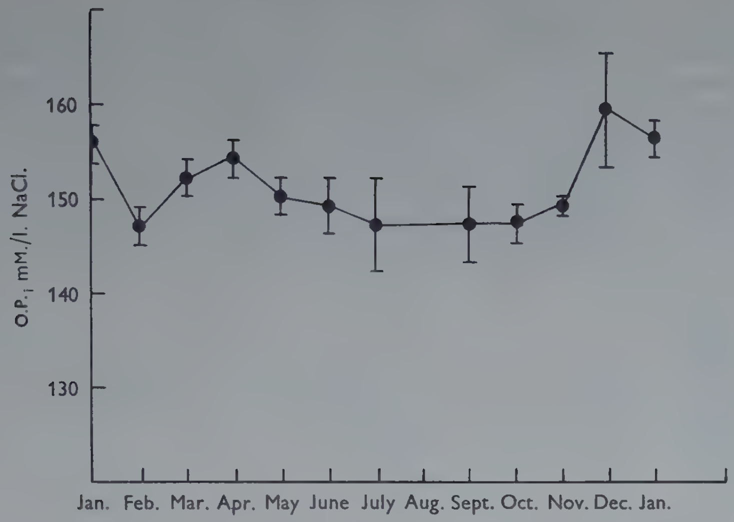

Fig. 5. Range and standard deviation of O.P.$_i$ month by month.

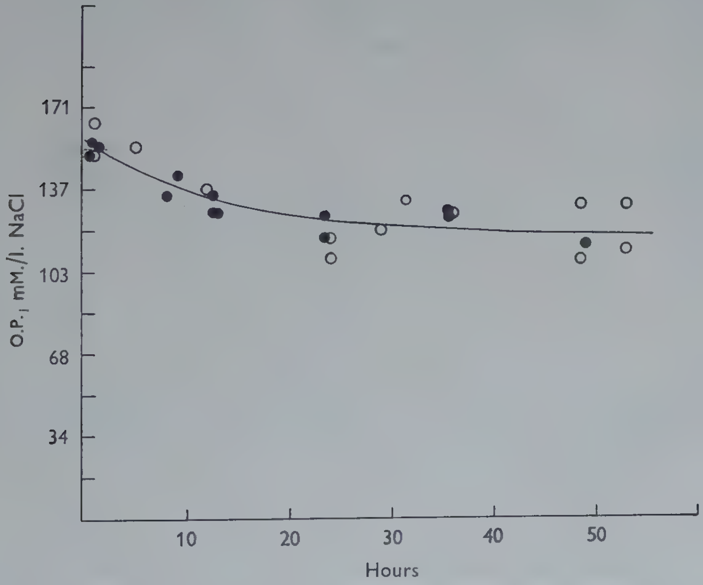

Fig. 6. Comparison of the rate of fall in O.P.$_i$ of animals washed with deionized water at different temperatures. ●, O.P.$_i$ of animals washed out at 24° C.: ○, O.P.$_i$ of animals washed out at 1° C.

(*b*) *The rate of sodium loss at different temperatures*

Two batches of animals were acclimatized for 36 hr. to temperatures of 1 and 24° C. and each batch was then exposed to a slow stream of deionized water at the temperature to which it had been acclimatized. Haemolymph samples were taken at intervals and the rates of fall of O.P.$_i$ at the two temperatures were compared. No significant difference was detectable between the rates (Fig. 6). Since Na$_i$

forms a constant proportion of O.P.$_i$ under a wide range of conditions (Lockwood, 1959*a*), this result suggests that the rate of ion loss is not affected by temperature. This conclusion was checked by washing out single fully loaded individuals successively at different temperatures and Fig. 7 illustrates the results of one such experiment. After loading, this animal was washed out at 16 ± 1° C. until its count had fallen to about 70% of the initial value. It was then allowed to take up sodium from the original loading medium and was finally washed out at 2 ± 1° C. The rates of loss of sodium were closely similar after the first few hours at the two temperatures. Similar experiments conducted over other temperature ranges indicate that the rate of loss of sodium is effectively independent of temperature (Fig. 8).

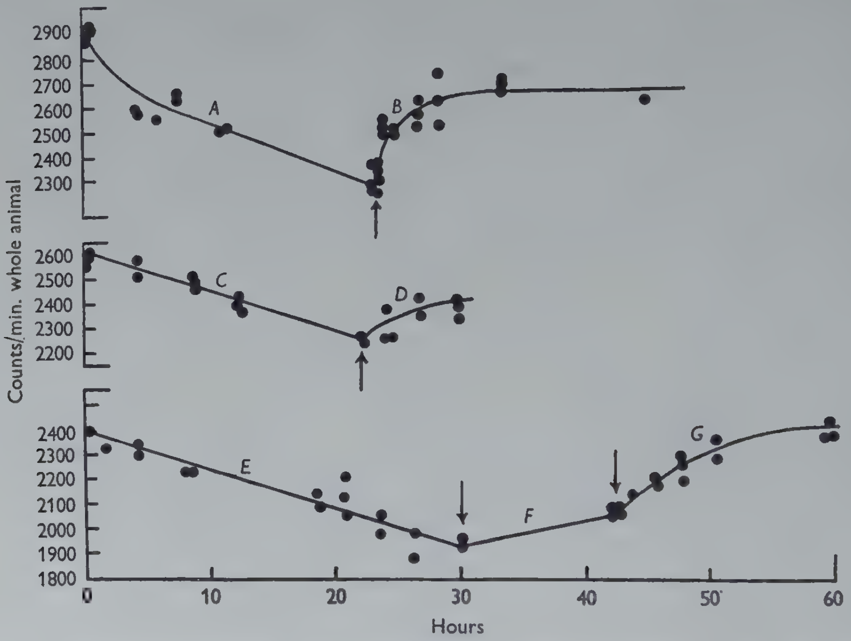

Fig. 7. The effect of temperature on the rate of loss and uptake of sodium. *A*, loss of sodium in deionized water at 16 ± 1° C. *B*, uptake from original loading medium at 16 ± 1° C. *C*, loss of sodium in deionized water at 2 ± 1° C. *D*, uptake from original loading medium at 2 ± 1° C. *E*, loss of sodium in deionized water at 2 ± 1° C. *F*, uptake from original loading medium at 2 ± 1° C. *G*, uptake from original loading medium when temperature was raised to 16 ± 1° C.

It is assumed that the values obtained for the initial rate of loss of sodium from the bodies of animals in deionized water correspond to the normal diffusion and urine loss. As might be expected there is some variation in the rate of loss. The mean rate found for the first 24 hr. of washing out fully loaded animals was $1{\cdot}00 \pm 0{\cdot}36\%$ of total body sodium per hour ($n = 20$). As some 20–30% of the total sodium is not in the haemolymph the mean rate of loss from the haemolymph would be expected to be of the order of 1·2–1·5%/hr. This agrees reasonably well with the initial rate of fall of the O.P.$_i$ of animals in distilled water of 1·6%/hr.

(Lockwood, 1959*b*) considering that the initial rate of loss of sodium is somewhat more rapid than the mean rate over 24 hr.

The half-time of net loss of haemolymph sodium, taking the rate of loss as being 1·6%/hr., would be 43 hr., whereas the half-time found for exchange of haemolymph sodium in the flux experiments was less than half this value (19–20 hr). The difference is presumed to be accounted for by the exchange diffusion component of the flux.

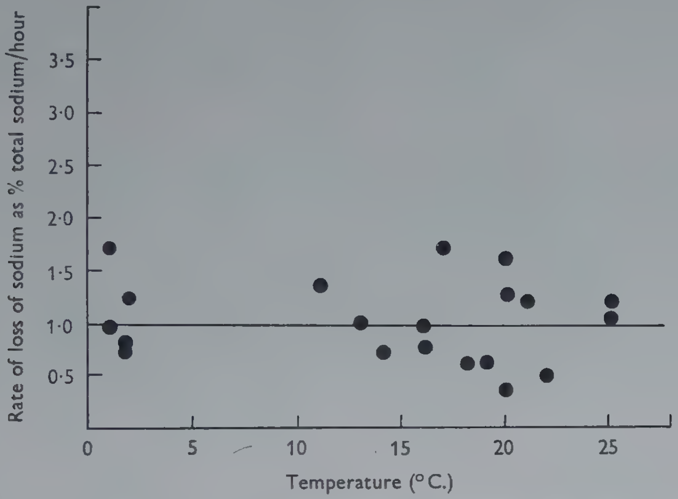

Fig. 8. Rate of loss of sodium (as % of total sodium/hr.) at various temperatures, during the first 24 hr. of washing out in deionized water.

(*c*) *The effect of temperature on the rate of active uptake of sodium by animals with a reduced haemolymph concentration*

Individual fully loaded animals were washed with deionized water at 1° C. until their count had fallen to a convenient level (70–50% of the initial count). They were then replaced in the loading medium (1·1 mM/l. NaCl) at 1° C. and allowed to take up sodium. After some hours the temperature of the medium was raised to 11° C. and the rate of uptake of sodium markedly increased. The animals were then washed out again at 11° C. to the same level as before and were finally replaced in the loading medium at 11° C. The course of one such experiment is shown in Fig. 9 and a comparison is made of the net rates of uptake at 1 and 11° C. in Fig. 10. In the experiment shown in Fig. 9 the initial net uptake at 11° C. is 2·0% of the initial total body sodium per hour or some five times as rapid as the net uptake at 1° C. which is only 0·4%/hr. However, in determining the temperature coefficient of the uptake mechanism account must also be taken of the sodium loss from the body during the period of uptake. In this case the rate of loss during the initial washing out phase was 1·3% of total body sodium/hr. The gross uptake of sodium at 11° C. is thus about twice that at 1° C., 2·0 + 1·3 = 3·3%/hr. as against 0·4 + 1·3 = 1·7%/hr.

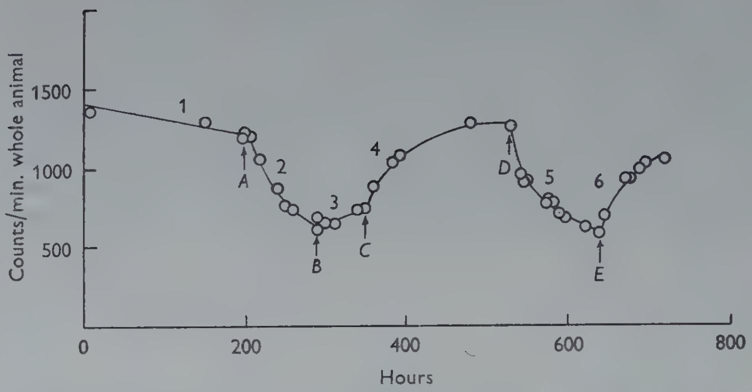

Fig. 9. The effect of temperature on the loss of sodium in deionized water and on the rate of uptake of sodium from a tracer medium. An animal was fully loaded with ^{22}Na at room temperature and at zero time the temperature was lowered to 1° C. The count rate of the whole animal fell while it was kept in the original loading medium at 1° C. (1). At *A* the animal was exposed to deionized water at 1° C. and suffered loss of sodium (2). At *B* it was replaced in the original loading medium at 1° C. and a slow uptake of sodium occurred (3). The temperature of the medium was raised to 11° C. at *C* and the rate of uptake increased (4), but declined again as the total sodium in the animal approached the normal level. At *D* the animal was transferred to deionized water at 11° C. and the loss of sodium (5) was similar to that during period 2. Finally, at *E* it was replaced in the tracer medium at 11° C. and uptake (6) was similar to that during period 4.

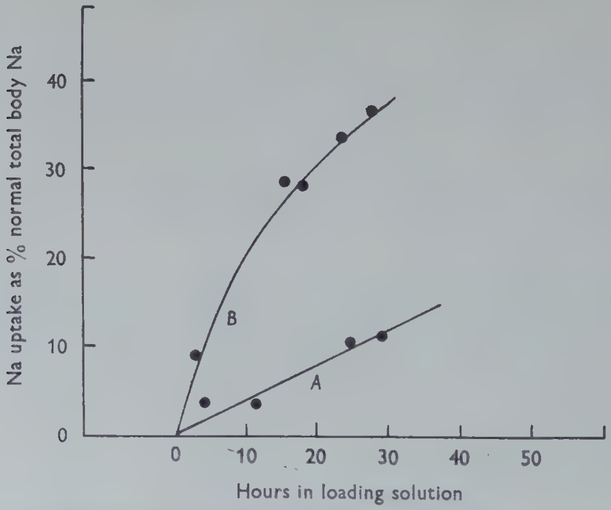

Fig. 10. The effect of temperature on the net rate of sodium uptake. A comparison of the rates of uptake at 1 and 11° C. by a single individual. *A* = uptake at 1° C., *B* = uptake at 11° C.

The maintenance of the haemolymph concentration at low concentrations of medium

Shaw (1959*a*) has shown that the rate of uptake of sodium by the crayfish is dependent on the concentration of the medium only when this is less than 1 mM./l. NaCl. At levels more concentrated than the critical level of 1 mM./l. NaCl the rate of uptake does not vary with the concentration of the medium. The critical level at which the concentration of the medium ceases to be a rate-limiting process is clearly of importance to fresh-water animals since any fall below this level must necessarily result in a drop in the steady-state haemolymph concentration.

A study has been made of the critical level in *Asellus*, but as this animal is too small for repetitive haemolymph sampling an indirect approach has been used. It is reasonable to assume that the concentration of the medium affects the carrier complex directly or indirectly in a manner independent of the mechanism which regulates the rate of uptake in response to changes in the Na_i. If the two processes are distinct then the critical concentration is the minimum concentration of the medium at which an animal can maintain its normal Na_i. The minimum Na_o at which *Asellus* can maintain its 'normal' Na_i was determined as follows. A number of animals were washed with deionized water until the O.P.$_i$ and Na_i of controls had fallen to about 100 mM./l. and 90 mM./l. respectively. Groups were then placed in various dilute solutions of NaCl in the concentration range 20–600 μM./l. After 7 days O.P.$_i$, Na_i and Na_o were determined. The two groups of animals in media less concentrated than 90 μM./l. had O.P.$_i$ and Na_i intermediate between the levels found in controls after the initial washing-out phase and the corresponding levels in normal untreated controls (Fig. 11). The critical concentration for the saturation of the carrier complex of *Asellus* is therefore of the order of 90 μM./l. at 19° C.

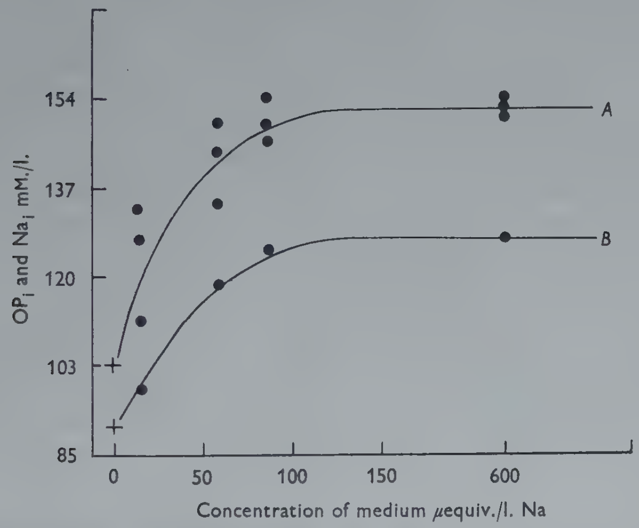

Fig. 11. Steady states of O.P.$_i$ and Na_i of animals in an external medium of low NaCl concentration. *A*, O.P.$_i$: *B*, Na_i: +, mean levels of O.P.$_i$ and Na_i of controls after the initial period of washing out in deionized water (see text).

From this experiment it may also be observed that the animal is able to maintain a steady-state Na_i at levels below the normal Na_i. Steady states are maintained only when loss of sodium is balanced by the active uptake of sodium. If the concentration of the medium falls below the critical level, uptake will show a corresponding decline. This decline is balanced, however, by the increased rate of uptake resulting from a fall in Na_i and a steady state is regained at a lower Na_i level than 'normal.' Interpretation of the results of the experiment in this manner involves the assumption that *Asellus* displays an increased rate of uptake when the Na_i falls below 'normal.' Such an effect has already been shown on a variety of species (Krogh, 1937; Shaw, 1959; Heuts, 1943), and has also been demonstrated on *Asellus*. This was done by washing fully loaded animals with deionized water and then replacing them in the medium in which they were originally loaded. The rate of uptake declines as the total sodium in the body approaches the 'normal' level. See, for examples, Figs. 7 and 9.

DISCUSSION

Asellus aquaticus has been shown to have only some 70–80 % of its total sodium in the haemolymph, much of the remaining 20–30 % being associated with uric acid in the cells of Zenker's organ (Lockwood, 1959*c*). The sodium content of these cells might be expected to have only a slow rate of exchange with the haemolymph, and it is therefore not surprising to find that the sodium flux in this animal is governed by two rate constants. It is presumed that the component of the flux responsible for 20–30 % of the total exchange and having the slow half-time of over 100 hr. at 19° C. is that of the sodium in Zenker's organ. The half-time of exchange of the other component of the flux has a value of about 20 hr. Rather few determinations have as yet been made on the sodium fluxes of arthropods but this latter value is intermediate between the values for exchange between haemolymph and external medium in the saline water crustacean *Artemia* (Croghan, 1958) and in the larva of the fresh-water mosquito *Aëdes* (Treherne, 1954).

The Na_i is maintained in a steady state when the active uptake of sodium is equal to the diffusion and urine losses. The rate of loss is slow (mean value 1·00 % total sodium/hr. or about 1·2–1·5 % Na_i/hr.) and fairly constant over the temperature range studied (1–24° C.). A transient increase in the rate of loss has sometimes been observed if the temperature is raised rapidly. The rate of loss of animals acclimatized to a given temperature is equivalent to a half-time of exchange of Na_i of 58–46 hr., that is, of the order of twice the value obtained in the corresponding flux experiments. The sodium influx from medium to haemolymph must therefore be separated into two components, exchange diffusion and active uptake. A similar exchange diffusion component of the sodium flux of arthropods has been shown by Croghan (1958) on *Artemia* and Stobbart (1959) on *Aëdes*.

If Na_i is lowered below the 'normal' value by washing an animal with deionized water the rate of active uptake of sodium is increased when the animal is returned to a normal medium. It is assumed that this increase in the rate of uptake is brought about by the mobilization of extra transporting sites.

Changing the temperature of the medium might be expected to result in a change in the rates of reactions involved in active transport and hence in the rate of operation of individual sites. The rate of active uptake of sodium by animals with a reduced Na_i displays such a temperature dependence, the uptake at 11° C. being about twice that at 1° C. Since the loss and uptake of sodium are not affected to the same extent by an alteration of the temperature of the medium the Na_i will also change. In fact, however, the change in the mean steady-state value of Na_i over the temperature range 8–24·5° C. is small, being only a few mM./l.

The small extent of this change in Na_i indicates the presence of a sensitive homeostatic mechanism and the following account describes a negative feed-back system which might act as the primary means by which the relative constancy of the Na_i is maintained under the influence of temperature change.

Lowering the temperature of the medium has the effect of decreasing the rate of active uptake of sodium. Since the rate of loss of sodium is fairly constant over a wide range of temperature such a fall in temperature has the effect of lowering the Na_i below the original steady-state level. But because the rate of active uptake increases as Na_i decreases, this fall below the 'normal' Na_i results in an increase in the rate of uptake of sodium. After a certain fall has occurred in the Na_i the rate of uptake is raised to a level at which it is again equivalent to the loss, and the Na_i will then again be in a steady state. The overall effect is thus a fall in the steady-state level of Na_i with decreasing temperature. Such a decrease in the concentration of the haemolymph as the temperature is lowered has been observed in *Gammarus pulex* as well as in *Asellus* (Heuts, 1943). Conversely, if the temperature is raised the steady state is regained at a higher concentration. It is clear that with such a mechanism regulating the Na_i under the influence of temperature the steeper the slope of the curve relating rate of uptake of sodium to the Na_i the smaller will be the change in the steady-state Na_i for any given temperature change. It is interesting therefore that Shaw (1959*a*) found that in the crayfish the rate of active uptake rises to a maximum when Na_i has fallen by only approximately 5–10%. In consequence this animal may be expected to be extremely well protected against the effects of temperature change.

In both *Asellus* and *Gammarus pulex* there is a marked rise in the haemolymph concentration at temperatures below about 4–5° C. (Heuts, 1943) and this appears to be incompatible with the suggested homeostatic mechanism outlined above. The experimental finding that the total sodium in the body is in fact reduced still further at low temperatures suggests that this effect is an artifact resulting from an accumulation of metabolites in the cells accompanied by a water shift from the haemolymph. However, despite this initial loss of sodium at low temperatures, a net uptake has been observed even at 1° C. in an animal with a low Na_i.

Shaw (1959*a*) has shown that in the crayfish the rate of active uptake of sodium is related to the concentration of the medium when this latter is less than about 1 mM./l. Above this critical value the rate of active uptake of sodium is independent of the concentration of the medium. In *Asellus* the corresponding critical value, obtained by an indirect method, is about 90 μM./l. NaCl—a level some eleven times less than that of the crayfish. A low critical value for the saturation of the carrier would seem to be of prime importance to a fresh-water animal in regions where the ion content of the water is low, and this may be one of the principal reasons for the well-known occurrence of *Asellus* in soft-water regions where the crayfish is not found. In Grasmere *Asellus* has been reported (Moon, 1957) from water only some 60 μM./l. Na above the experimentally determined critical value.

The high temperature coefficient of active uptake relative to that of loss found in *Asellus*, if more generally applicable, may have a wider physio-ecological significance. According to von Martens (1858) tropical and subtropical estuaries and fresh waters contain more quasi-marine species than do the corresponding temperate waters. In addition, a number of temperate brackish-water species have apparently given rise to very similar forms which live in fresh water near the warmer southern regions of the range of the parent species. Such forms include *Jaera balearica* (Margalef, 1952) and *Palaemonetes antennarius*. Various suggestions have been made in an attempt to explain the possible influence of high temperatures on the invasion of fresh water by brackish-water animals. These include the relative stability of temperatures in tropical fresh waters (von Martens, 1858) and a suggestion by Pannikar (1940) that the optimum osmotic pressure of the haemolymph may be lower at high temperatures. The present results suggest a further possibility, though caution must naturally be exercised in the application of conclusions derived from results based on a single fresh-water species to more general considerations. If the range of a brackish-water species is limited by the minimum Na_i and Cl_i at which it is sufficiently physiologically normal to compete effectively in its environment, then it can achieve these concentrations in a dilute medium more readily if the temperature is high than if it is low. A corollary of this is that for a given limiting haemolymph concentration penetration will be further towards fresh water the warmer the medium. Some support for this contention is provided by the fact that in very dilute media *Asellus* maintains a higher Na_i at high temperatures than it does at low temperatures, and by Broekhuysen's (1936) finding that the eggs of *Carcinus maenas* would develop at a salinity of 20‰ at 16·3° C., but could not tolerate less than 26‰ when the temperature was lowered to 10° C. Similarly, Broekema (1941) found than *Crangon crangon* survived low salinities better when the temperature was high than when it was low, and he discusses this in relation to the seaward migration of this animal at the beginning of winter. Shaw (1959*b*) has shown that the fresh-water crab *Potamon niloticus* loses sodium to the medium when the temperature is lowered and he points out that this species is not found in the colder upper reaches of the East African rivers.

However, high temperatures do not assist all brackish water species to move towards more dilute waters. *Gammarus duebeni* may be cited as an example of a species in which the temperature coefficients of loss and uptake of sodium bear an inverse relationship to those found in *Asellus* (unpublished observations). This is also supported by recent work by Kinne (1959) on the survival of this animal at different temperatures and salinities. Animals in this category might be expected to be more likely to invade fresh water in cold regions.

SUMMARY

1. Some effects of temperature and concentration of the medium on the sodium metabolism of the isopod *Asellus aquaticus* have been studied.

2. The steady-state haemolymph concentration rises with the temperature in the range 5–24° C. The change is, however, only a few mM./l. over this temperature range. The osmotic pressure (O.P._i) and sodium concentration of the haemolymph (Na_i) also rise at temperatures below about 4–5° C.

3. The rate of sodium loss from the body is unaffected by temperature.

4. The rate of active uptake of sodium is increased as the temperature is raised.

5. The rate of active uptake of sodium is related to the haemolymph concentration, rising as the Na_i falls.

6. The Na_i is maintained at the normal level in media containing more than 90 μM./l. NaCl. Steady states can also be maintained at reduced haemolymph concentrations in media less concentrated than 90 μM./l.

7. It is suggested that the homeostatic mechanism regulating the Na_i under the influence of temperature operates as follows. As the temperature falls there is a greater decrease in the rate of uptake than in the rate of loss and hence the Na_i falls. A fall in the Na_i, however, results in a rise in the rate of uptake. Therefore after a certain fall in Na_i the loss is again balanced by uptake and a new but lower steady state is reached.

8. It is tentatively suggested that high temperatures may assist some brackish-water species in the invasion of more dilute media.

I would like to thank Dr P. C. Croghan for many helpful discussions. I am indebted to the Department of Scientific and Industrial Research for a maintenance grant and the Earl of Moray Endowment for the Promotion of Original Research for a grant for instruments.

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ASPECTS OF FLIGHT MECHANICS IN ANISOPTEROUS DRAGONFLIES

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I. INTRODUCTION

Our understanding of the flight mechanics of dragonflies is very incomplete. Many accounts have been given of the relevant skeletal structures and musculature (e.g. Chao, 1953; Clark, 1940; Cremer, 1934; Tannert, 1958), and a number of the more obvious flight parameters have been determined experimentally (e.g. Magnan, 1934; Chadwick, 1940). But assertions as to the movements of various skeletal structures in the wing-base and pterothorax, and attempts to relate these to muscular contractions are little more than inferences from anatomical data. Moreover, even these data show many discrepancies when the various published accounts are compared, and some of the descriptions are known to be inaccurate. The main purpose of this paper, therefore, is to report direct observations on the movements of various skeletal structures in the pterothorax and wing-base during flight, to analyse some of these movements quantitatively, and to determine by experimental transections the precise role played by various muscles in determining the movements of the sclerites and therefore of the wings themselves.

The mechanics of insect flight involve two main sets of processes. The first is concerned with the flight power muscles and the leverages involved in elevating and depressing the wing, while the second concerns the movements of pronation and supination of the wing. Weis-Fogh (1956*a*) has emphasized that the aerodynamic lift on the wing is adjusted by means of these changes in rotation (i.e. pronation and supination) since they alter the angle of attack of the wing. It is the changes in rotation which are the subject of this paper; the other main aspect of the problem will be discussed elsewhere.

So far, changes in rotation have been shown to occur by rather different mechanisms in different groups of insects. Thus Jensen (1956) showed in *Schistocerca gregaria* Forskål that changes in aerodynamic section of the forewing during flight are actively controlled from the wing-base, and Weis-Fogh (1956*a*) demonstrated that this results from the control of rotation by the main flight power muscles. In *Sarcophaga*, however, Boettiger & Furshpan (1952) have shown how wing twisting is co-ordinated into the wingstroke cycle at the correct phase solely by relative movements of the basal articulations. Again, in some Hymenoptera Pringle (1957) found that pronation and supination are correctly phased into the stroke through the arrangement of 'indirect' muscles and axillary and thoracic sclerites.

The present work will show that in Odonata the twisting of all four wings is controlled by small accessory flight muscles. Whereas in *Schistocerca* both flight power and rotational changes are provided by the same muscles, in the dragonflies there is a division of labour in that the larger muscles are used only for flight and the rotation is controlled by smaller ones. The system in dragonflies may therefore be regarded as a refinement of that represented in the locust.

II. TERMINOLOGY, MATERIAL AND METHODS

(*a*) *Terminology*

The terms used to describe the various flight parameters are defined by Weis-Fogh & Jensen (1956). The meaning of the more important of these will be apparent from the account of methods of measurement given below in section (*c*).

The morphology of the muscles, sclerites and ligaments of the dragonfly thorax has been revised and will be dealt with by Neville and Weis-Fogh elsewhere. For most of the better-known sclerites, the terminology of Chao (1953) is followed, but the following notes on some of Chao's terms indicate the usage employed here:

Adnotal sclerite: this is here regarded as part of the posterior lobe of the lateroprescutum.

Anterior notal wing process: here regarded as part of the posterior lobe of the lateroprescutum.

Axillary plate: here called the axillary complex, it is regarded as homologous with the fused first, second, third (and perhaps fourth) axillary sclerites of other insects, together with the subalare and the bases of the veins R + M, Cu 2 and 1 A.

Humeral plate: here called the humeral complex, it is regarded as homologous with the first and second basalares, the true humeral plate and two other sclerites.

Interalary sclerite: this seems to be the tegula of the metathorax.

Prealare sclerite: this is regarded as part of the anterior lobe of the lateroprescutum.

The nomenclature of the muscles follows Clark (1940) with the following changes:

Small first basalar muscle: now regarded as the first basalar.

Large second basalar muscle: now regarded as the second basalar.

Subalar muscle: now regarded as the first subalar depressor.

Pleuroaxillary muscle: now regarded as the second subalar depressor.

Pleurocubital muscle: now regarded as the third subalar depressor.

(*b*) *Material*

Adult material of *Aeshna cyanea* (Mueller) and *A. juncea* (L.), was collected from Tisvilde Hegn and Hilleröd Store Dyrehavre, North Zealand, Denmark. The larger species, *A. cyanea*, was particularly useful for quantitative studies of wing-base and notal sclerite movements. Males were used throughout the experiments, as they were easier to collect and were more willing fliers than gravid females.

The insects were stored in tightly closed polythene boxes in a refrigerator at 10° C. Deaths through desiccation were overcome by lining the walls of the boxes with wet filter-paper (Miller, 1959). The insects were only brought into room temperature (21–24° C.) as required for experiments, since long periods spent flapping actively at this temperature greatly reduce the survival time. The insects were fed once a week on larvae of *Musca domestica* L. This also was performed as quickly as possible at room temperature. Altogether, sixty-six adults were used in the experiments.

(*c*) *Methods*

(i) *Wind tunnel*

The insects were flown 10 cm. in front of the centre of windflow of an open jet wind tunnel (Fig. 4), which is fully described by Weis-Fogh (1956*a*). Wind-speeds were calibrated with a Pitot static tube and Fuess manometer with sloping scale as described by Jensen (1954). The calculation of the wind-speed was that given in Irminger & Nökkentved (1930). A wind-speed of 2·5 m./sec. was used throughout the experiments.

(ii) *Stroboscopic observation*

A General Radio Co. 'Strobotac' was used for stroboscopic studies of wing-base and notal sclerite and wing movements, in conjunction with a concave shaving mirror to focus a suitable concentration of light on to the wing-base of the flying dragonfly. The light intensity was adequate for use with a × 20 Leitz 'Wetzlar' binocular microscope; measurements were made with a micrometer eyepiece. A certain amount of continuous light was used to make the dragonflies fly, and was place dorsally above them (Fig. 4). The insects could thus be observed at a convenient angle with the microscope, whilst the dorsal light ensured equal wing movements on either side; this makes use of the dorsal light reaction (von Buddenbrock, 1937; Mittelstaedt, 1950). This illumination also helped in lighting up the object, and thus the micrometer scale also, as all observations were necessarily by reflected light; the intensity was adjusted so as not to cancel out the stroboscopic light and to make movements appear continuous. White spots of cellulose paint were used on various sclerites: these showed up clearly on the micrometer scale. When wing rotation was being observed stroboscopically, a fine hair was painted and fastened to the wing with white cellulose paint, at the region of the pterostigma (Fig. 1), where the changes in angles of rotation are greatest (Weis-Fogh, 1956*b*). Errors in measurement and observation would then be at a minimum.

(iii) *Suspension and measurements*

Rigid suspension was used, so that the movements of sclerites could be measured relative to the insect and not to any external reference points. For initial qualitative observation, a simple brass rod with a small square end-piece was constructed. The insect was glued to this by the metasternum with the standard mixture of beeswax and colophonium resin (Wooten & Sawyer, 1954). For quantitative measurements the rigid locust suspension devised by Weis-Fogh (unpublished) was modified with a piece of cork to suit *Aeshna* (Fig. 2). The prism in this suspension translates into horizontal movements vertical components of movements of the sclerites. Horizontal components of sclerite movement were measured directly (i.e. not through the prism). Thus, during the downstroke and with the wind tunnel to the left, a spot on the scutum moves from left to right under the microscope. Conversely, during the upstroke, the spot moves from right to left (Fig. 3). Also, considering the vertical movement of the scutum, on the downstroke the spot moves up, again from left to right as seen through the dorsal surface of the prism: and during the upstroke, the spot moves down, from right to left through the prism. So, in slow motion, during the downstroke, the scuta move to the right and during the upstroke to the left. The half-cycles are thus distinguishable in stroboscopic slow motion through the microscope. The insects were flown at a constant body angle of 7° above the horizontal. Measurements of wing position Γ were made from behind the animal and are expressed as degrees from the lower vertical about the longitudinal body axis (Weis-Fogh & Jensen, 1956). Rotation angles were measured with a protractor in plane projection on the median longitudinal (sagittal) plane; the arrangement of the apparatus is shown in Fig. 4.

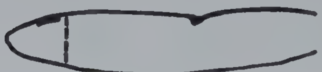

Fig. 1

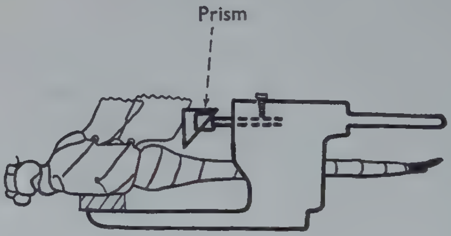

Fig. 2

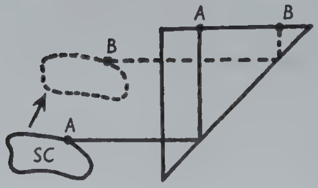

Fig. 3

Fig. 1. Diagram to show the position of a fine hair attached to the forewing of *Aeshna juncea*, 9 mm. from the wing-tip.

Fig. 2. Diagram of Weis-Fogh's locust suspension and prism, modified for *Aeshna*. Vertical movements of the wing-base and notal sclerites can be measured through the prism. The suspension is shown at zero body angle, but was adjusted to 7° during experiments.

Fig. 3. Diagram to show the measurement of the vertical movement of the scutum (*SC*), from *A* to *B*, through the prism (sagittal view).

(iv) *Flight initiation and posture*

Dragonflies are temperamental fliers; the longest bursts of consistent flight obtained were no more than 15 min. They were induced to fly by the tarsal reflex system, which does not function to give an 'all or none' reaction as in most insects. Removal of tarsal contact does not always induce flight, nor do dragonflies usually cease flight on regaining tarsal contact. The mechanisms involved are worth investigation. When loss of tarsal contact failed to start flight, pinching the abdomen, and in particular the terminalia, was usually successful, but not always. Visual stimulation by means of moving a pencil up and down above the eyes was tried as a means of flight excitation, but was unsuccessful. The posture during steady flight is shown in Fig. 5, but the legs were frequently extended and waved through the air in a typical prey-catching action.

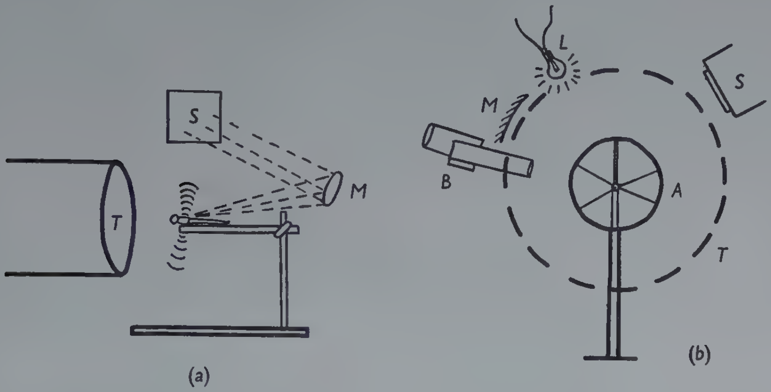

Fig. 4. Diagram showing the apparatus for observing sclerite movements during flight; (*a*) left lateral, and (*b*) posterior view. The insect faces the wind tunnel orifice, *T*, and is illuminated by the small dorsal light, *L*, so that it can be flown at an easily observable angle with symmetrical wing movements. The light from the stroboscope, *S*, is condensed on to the wing-base by the concave mirror, *M*, making possible observation through the binocular microscope, *B*. *A* is an instrument for measuring the positional angle of all four wings.

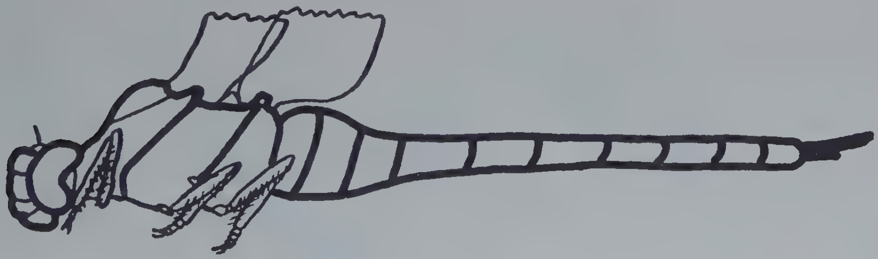

Fig. 5. The flight posture in *Aeshna* (left lateral view).

III. THE MOVEMENTS OF THE WING-BASE AND NOTAL SCLERITES DURING FLIGHT

Using the technique described, the movements of the tergal and wing-base sclerites were observed from the dorsal aspect, at 22° C. and 2·5 m./sec. wind-speed, stroke frequency 35 per sec.

(*a*) *Scuta and scutella*

The sequence of movements occurring here is shown in Fig. 6. At the commencement of the upstroke of the hindwings (Fig. 6*A*), the metascutum begins to move downwards and forwards, causing the metascutellum, to which it is fused, to move with it. The movement continues forwards along the pterothoracic nota with an evident phase difference between the two wing segments. The mesoscutum begins to move forwards and downwards shortly after the metascutum, causing the mesoscutellum, to which it is fused, to move with it. The metascutum also pulls the mesopostnotum forwards and down, the posterior end of the latter sclerite moving before the anterior end (Fig. 6, *B*).

The role of the tergosternal muscles which cause the above movements was illustrated in an experiment in which the dorsal light was placed to the left of the dragonfly. The insect then turned its head to the left and an attempted rolling action occurred. The right dome of the metascutum was now seen to be moving down on the upstroke to a much greater degree than the left dome, indicating a

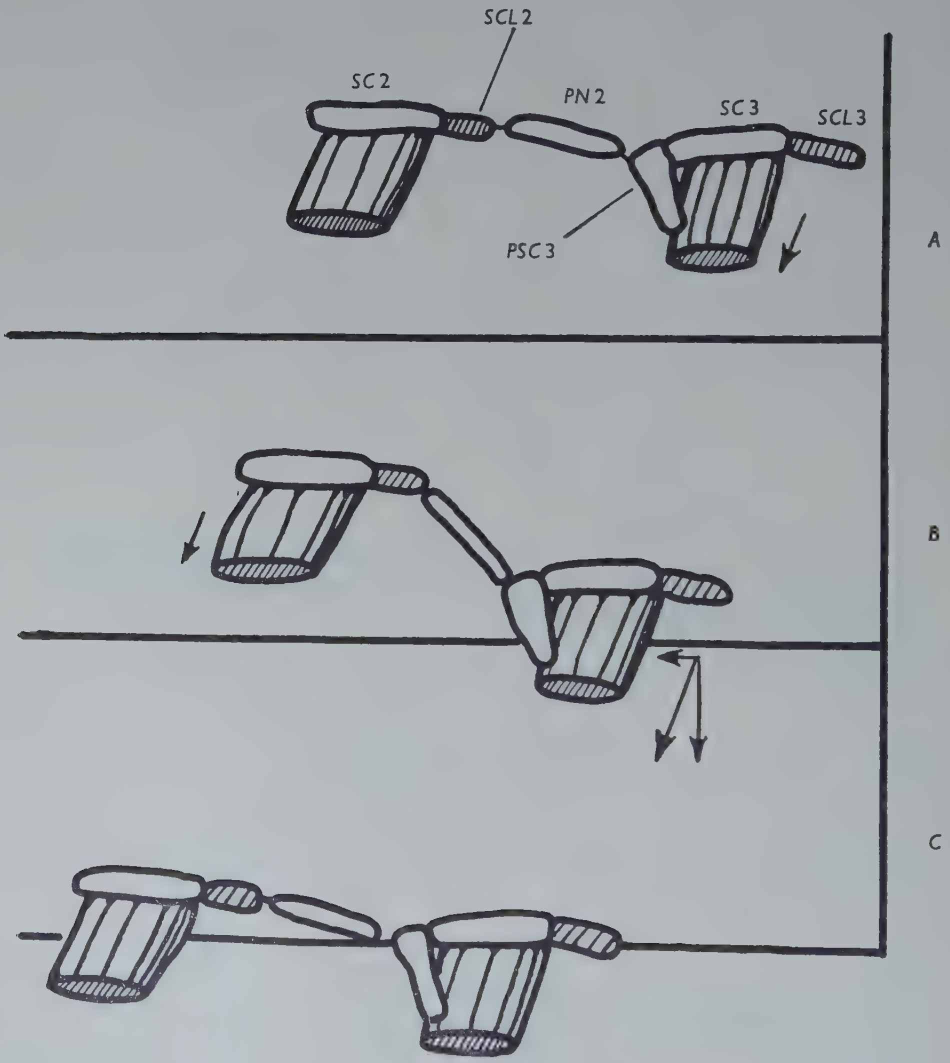

Fig. 6. Diagrams drawn at successive stages of the upstroke, showing the movement of the metascutum (*SC* 3), and metaprescutum (*PSC* 3), which begin to move downward and forward before the mesothoracic sclerites *SC* 2 and *SCL* 2. This results in the antero-posterior tilting of the mesopostnotum (*PN* 2). Movements are denoted relative to the fixed horizontal and vertical axes. *A*, beginning of upstroke: *B*, middle of upstroke: *C*, end of upstroke.

weaker contraction of the muscles of the left-hand side. This resulted in the stroke angle of the left wings being reduced. Similarly, in the mesothorax, the single-domed mesoscutum also moved down more on the right-hand side, but not so noticeably as the metascutum, probably because of the greater width of the latter.

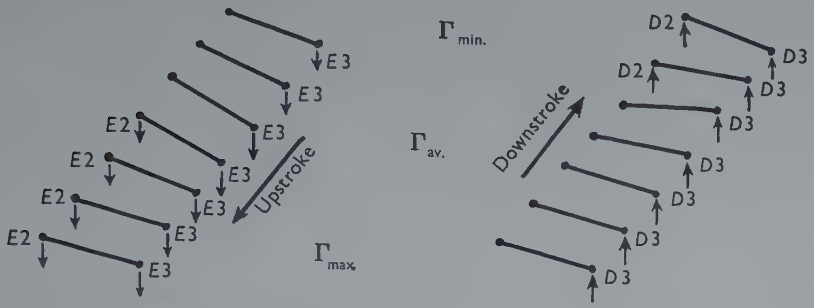

Fig. 7. The movement of the mesopostnotum throughout the wingstroke from horizontal and vertical stroboscopic observations. *E* 2, direct force from mesothoracic elevators transmitted via the mesoscutum. *E* 3, direct force from metathoracic elevators transmitted through the metascutum. *D* 2, indirect force from the mesothoracic depressors plus the forces from the pterothoracic cuticle and elastic ligaments, transmitted through the mesoscutum and mesoscutellum. *D* 3, indirect force from the metathoracic depressors plus forces from the pterothoracic cuticle and elastic ligaments, transmitted through the metascutum and metaprescutum. Phase difference is incorporated into this diagram.

(*b*) *Mesopostnotum*

Shortly after the beginning of a normal upstroke, the distance between the posterior end of the mesoscutellum and the metaprescutum is reduced. This compression effect, caused by the small forward component in the movement of the metascutum, moving before the mesoscutum begins to move, is allowed for by means of the antero-posterior tilting of the mesopostnotum. There are no muscle insertions on the latter sclerite. Its movement during the upstroke is the result of the two forces mentioned above; it moves downwards and forwards initially with a backward tilt which is then levelled off again (Fig. 7). During the downstroke the sclerite is acted upon indirectly by the force from the metathoracic depressors through the metascutum and metaprescutum, and then slightly later by the mesothoracic depressors via the mesoscutum and the mesoscutellum.

(*c*) *Anterior lobe of the lateroprescutum*

This sclerite was painted, and the horizontal and vertical movements were measured during normal flight (Table 1). The results are plotted in Fig. 9 and the movement of this sclerite is shown qualitatively in Fig. 8. The posterior end of the sclerite is pulled downwards (angle of movement 38° from vertical) more than the anterior end (47° from vertical), by the contraction of the anterior coxoalar muscle. This movement was seen to correspond with supination during the last part of the downstroke and during the upstroke. The anterior end is fused to the prescutum and so moves as a unit with the scutum. The latter sclerite was found to move at an angle of 48° to vertical under the same conditions.

Table 1. *The movement of the anterior lobe of the lateroprescutum*

Point measured	Wings full up (mm.)		Wings full down (mm.)	
	Hor. comp.	Vert. comp.	Hor. comp.	Vert. comp.
Posterior end	2·49	3·16	2·31	2·98
Anterior end, just posterior to anterior articulation	2·49	3·11	2·31	2·98

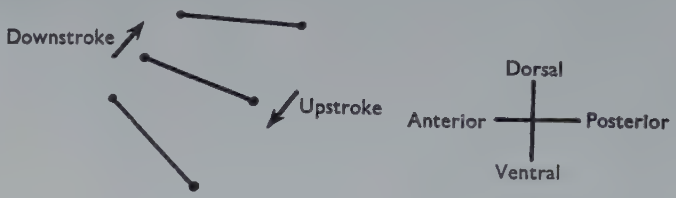

Fig. 8. The movement of the anterior prong of the lateroprescutum throughout the wingstroke, from qualitative horizontal and vertical stroboscopic observations.

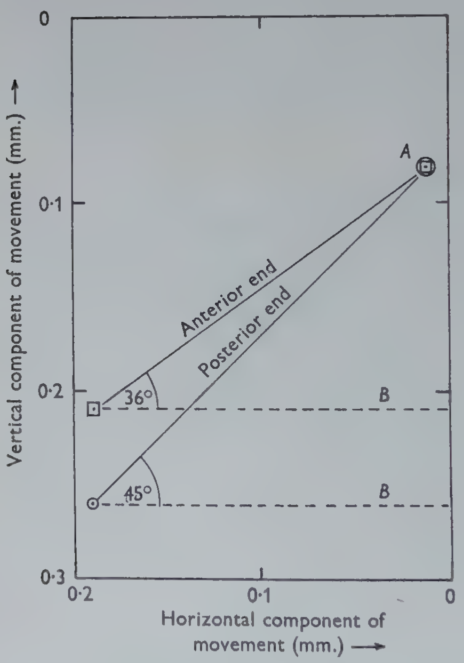

Fig. 9

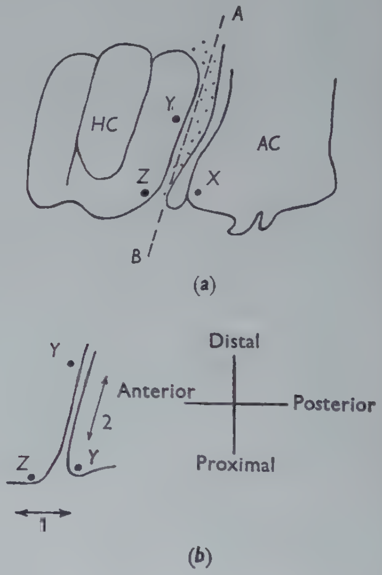

Fig. 10

Fig. 9. Graph of the movement of the anterior end (squares) and the posterior end (circles) of the anterior prong of the lateroprescutum in a male *A. juncea*. From quantitative measurements. Temperature 22° C. Wind-speed 2·5 m./sec. Full flight. N 35/sec. Body angle 7°. Therefore true angle of movement of the anterior end is $36^\circ + 7^\circ = 43^\circ$: and of the posterior end, $45^\circ + 7^\circ = 52^\circ$ *A*, forewings full down at $\Gamma_{\text{min.}}$: *B*, forewings full up at $\Gamma_{\text{max.}}$.

Fig. 10. (*a*) The relative movements of the humeral complex (*HC*) and axillary complex (*AC*), about the line of weakness *AB*. The white paint spots, *X*, *Y* and *Z* are shown in (*b*), together with the relative movements of the sclerites, which may be resolved into two components, movement 1 (antero-posterior) and movement 2 (proximal-distal).

Pushing down on the anterior lobe of the lateroprescutum with a fine needle in the line of action of the anterior coxoalar muscle on a relaxed dead specimen resulted in supination of the wing. Elevation occurred during this manipulation: this muscle acts synergically to give elevation with the first tergosternal muscle and the posterior coxoalar muscle, which inserts on the posterior lobe of the lateroprescutum.

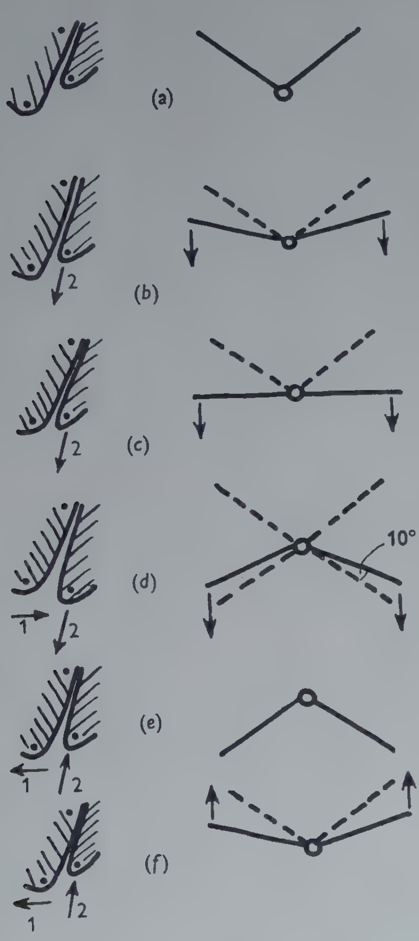

Fig. 11

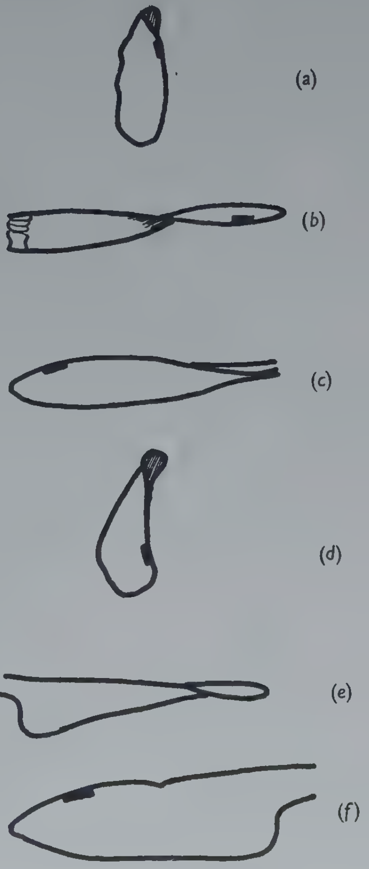

Fig. 12

Fig. 11. Sequence diagrams showing the relative movements 1 and 2 (see Fig. 10). (*a*) Wings full up: (*b*), (*c*), (*d*), downstroke: (*e*) wings full down: (*f*) upstroke. The sudden snap inwards and backwards of the axillary complex is seen in (*d*). (*e*) shows that the axillary complex has started to return outwards and forwards before the end of the downstroke. From qualitative stroboscopic observations.

Fig. 12. Diagrams to show the complex supination section resulting from manipulation by downward pressure on the anterior edge of the axillary complex. (*a*), (*b*), (*c*), Forewing; (*a*) Right forewing, end-on view: (*b*) dorsal view of right forewing: (*c*) anterior view of right forewing: (*d*), (*e*), (*f*), hindwing. (*d*) Right hindwing, end-on view: (*e*) dorsal view of right hindwing: (*f*) anterior view of right hindwing.

(*d*) *The humeral complex and the axillary complex*

The supination movement in *Aeshna* occurs about a line of weakness running between the humeral complex (humeral plate) and the axillary complex (axillary plate) (Fig. 10). Accordingly, the relative movement of these two sclerites about the line of weakness was investigated. For this, *A. cyanea* was used, as it is a better flier than *A. juncea* and also larger and therefore easier to observe. Spots of white paint were placed on the humeral complex at *Y* (laterad) and *Z* (mesad). A spot, *X*, was placed on the axillary complex, as shown in Fig. 10. Qualitative stroboscopic observations showed that the relative movements of these two plates could be resolved into two horizontal components: movement of *Z* from *X* was denoted movement 1, and movement of *X* from *Y* as movement 2. Movement 1 (see Fig. 11) occurs suddenly with a snap action at 10° from the bottom of the forewing downstroke, simultaneously with the beginning of supination and presumably at the beginning of contraction of the anterior coxoalar supinator muscle. Also, at this instant during the downstroke, the posterior end of the anterior lobe of the lateroprescutum begins to be pulled down by the contraction of the same muscle (see below). This sharp snap action does not, of course, occur when the wings are manipulated in a freshly killed specimen. Before the quantitative results of movements 1 and 2 can be interpreted, it is first necessary to consider the movements of the anterior lobe of the lateroprescutum during flight.

Table 2. *Vertical movement of the anterior lobe of the lateroprescutum relative to the humeral complex*

Γ	Downstroke (mm.)	Upstroke (mm.)
118° 30′	0·089	0·089
80°	0·089	0·222
71° 30′	0·156	0·156

The vertical movement of this sclerite relative to the humeral complex was measured. Both sclerites were marked with white cellulose paint and measurements made through the prism during normal flight. For convenience, the humeral complex is considered as stationary, while the movements of the anterior lobe of the lateroprescutum relative to it in the vertical plane are plotted against wing position Γ (Table 2 and Fig. 13). Actually, the proximal end of the humeral complex moves up during the downstroke and down during the upstroke. It was found that the anterior lobe of the lateroprescutum began to move ventrally from the humeral complex when the wing position Γ was 80° on the downstroke, whereas Γ minimum was 71° 30′. Thus, the anterior coxoalar elevator muscle, which inserts on the posterior lateral margin of the anterior prong, begins to contract during the last 10° of the downstroke. The sclerite reaches its maximum ventral position at $\Gamma = 80°$ on the upstroke (Fig. 13). Movements 1 and 2 were also investigated during a manipulation wingstroke for comparison with a normal wingstroke (Table 4).

We are now in a position to understand the quantitative measurements of movements 1 and 2 which are given in Table 3. During a manipulation downstroke, passive promotion of the wing relative to the wing-base must inevitably occur, because of the forward and downwardly-pointing stroke plane. There is therefore a gradual movement apart of the humeral and axillary complexes (movement 1), but this is not, of course, accompanied by the sudden snap action at $\Gamma = 80^\circ$, produced by the anterior coxoalar muscle during a normal wingstroke (Fig. 14). In manipulation, remotion in the initial stages of the upstroke is accompanied by the relatively abrupt movement towards each other of the humeral and axillary complexes. The same movement in a normal wingstroke is smoother and more gradual, implying that the return together of the sclerites is controlled by the anterior coxoalar muscle. Over the rest of the upstroke and beginning of the downstroke, the two graphs are similar, as passive remotion relative to the wing-base occurs because of the backward and upwardly pointing stroke plane.

Table 3. *Relative movements of the axillary complex about the line of weakness between it and the humeral complex*

Wing position Γ	Posterior deviation of X from Z during wingstroke (movement 1)	
	Downstroke (mm.)	Upstroke (mm.)
Maximum 120°	0·089	0·089
Minimum + 10° = 80°	0·133	0·266
Minimum 70°	0·311	0·311
	Inward deviation of X from Y during wingstroke (movement 2)	
Wing position	Downstroke (mm.)	Upstroke (mm.)
Maximum 120°	0·445	0·445
Minimum + 10° = 80°	0·622	0·711
Minimum 70°	0·80	0·80

Table 4. *Passive movements between the humeral complex and the axillary complex during wing manipulation*

Γ Wing position	Movement 1	
	XZ	XZ
	Before remotion	After remotion
Minimum 70°	0·225 mm.	0·089 mm.
	Before promotion	After promotion
Maximum 115°	0·045 mm.	0·045 mm.
	Movement 2	
Wing position	XY	XY
	Before remotion	After remotion
Minimum 70°	0·445 mm.	0·222 mm.
	Before promotion	After promotion
Maximum 115°	0·089 mm.	0·178 mm.

For similar reasons, movement 2 also occurs during a manipulation downstroke (Fig. 15), and again the snap observed in a normal downstroke at $\Gamma = 80^\circ$ is absent. Again the return together of X and Y is controlled by the anterior coxoalar muscle during the normal stroke, but occurs abruptly during manipulation remotion.

Another sudden movement apart at $\Gamma = 115^\circ$ and causing wing promotion, occurs only during manipulation. The more gradual movement in the normal downstroke must therefore presumably be controlled by the second subalar depressor supinator muscle, whose function will be dealt with later. Figs. 14 and 15 also show that movements 1 and 2 are larger during a normal wingstroke than in manipulation.

It is convenient now to sum up the transmission of the supination force from the anterior coxoalar muscle to the wing. The muscle pulls on the distal, posterior free edge of the anterior lobe of the lateroprescutum, during the last 10° of the down-

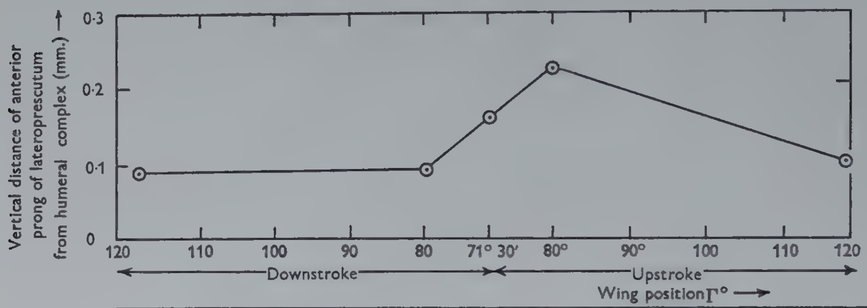

Fig. 13

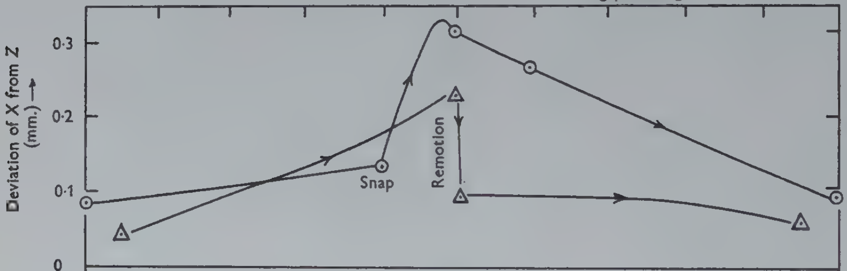

Fig. 14

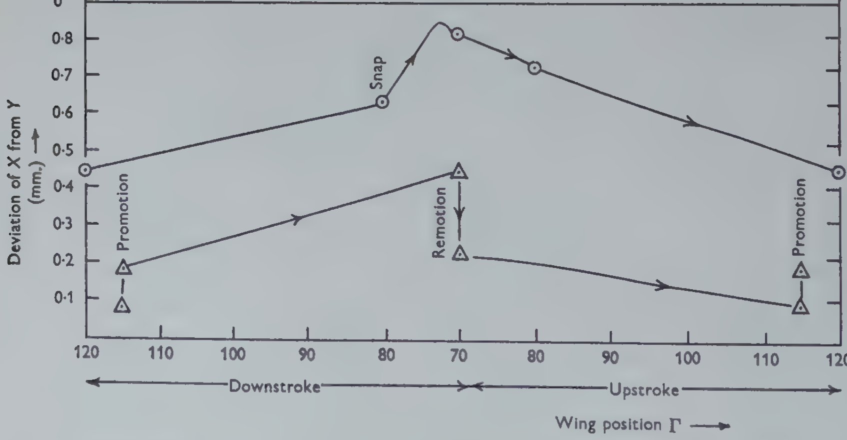

Fig. 15

Fig. 13. Quantitative graph of the relative vertical movement of the anterior prong of the lateroprescutum from the humeral complex, during the wingstroke.

Fig. 14. Comparison of the relative movements (movement 1) between the humeral and axillary complexes during a normal wingstroke (circles) in *A. cyanea*, and a manipulation wingstroke (triangles) in *A. juncea*.

Fig. 15. As for Fig. 14, for movement 2 (same horizontal axis as for Fig. 14).

stroke and throughout the upstroke, when it also functions as an elevator muscle. Force is transmitted across the dorsal wing membrane on to the posterior edge of the humeral complex. The wing takes on a complex supinated section, diagrams of which are given in Fig. 12. The force enters the wingstroke with a visible snap action, which is not seen in manipulation experiments. The fact that it is not demonstrates that supination is a process which is actively controlled in the flying insect by muscular contraction.

(*e*) *Scutal ascension*

In *Schistocerca* Weis-Fogh (unpublished) found that at the mid-position of Γ the scutum was higher on the downstroke than on the upstroke. The difference he called 'scutal ascension'. This was detected also in the dragonfly by careful adjustment of the stroboscope so that the upstroke could be distinguished from the downstroke. The wing was observed going down in slow motion with the stroboscope frequency just below fundamental synchronization. The stroboscope was then adjusted to fundamental synchronization to stop the wing at Γ average (downstroke). Readings of vertical and horizontal components of scutal movements were then taken, and the whole process was repeated for Γ average upstroke, Γ minimum and Γ maximum. Results for the movements of mesoscutum, mesopostnotum and metascutum are given in Table 5. These results are plotted for movement of mesoscutum (Fig. 16), for mesopostnotum and metascutum (Fig. 17).

Table 5. *Horizontal and vertical movements of the scuta and mesopostnotum during the wingstroke*

Wing position	Component	*SC*2	*SC*2	*PN*2	*PN*2	*SC*3
$\Gamma_{\text{av.}}$ upstroke	Horizontal	2·66	0·98	3·47	2·47	1·89
	Vertical	2·33	1·78	2·34	2·78	2·38
$\Gamma_{\text{av.}}$ downstroke	Horizontal	2·62	0·934	3·44	2·45	1·87
	Vertical	2·31	1·73	2·31	2·75	2·36
$\Gamma_{\text{max.}}$ 115°	Horizontal	2·89	1·16	3·55	2·53	1·96
	Vertical	2·40	1·86	2·4	2·84	2·44
$\Gamma_{\text{min.}}$ 70°	Horizontal	2·44	0·845	3·33	2·36	1·78
	Vertical	2·17	1·64	2·22	2·67	2·31

Key. *SC*2, mesoscutum; *SC*3, metascutum; *PN*2, mesopostnotum (all measurements in mm.).

These graphs show that at the mid-wing position average Γ, the mesoscutum, metascutum and mesopostnotum are all higher on the downstroke than on the upstroke. They are also more posterior on the downstroke. Fig. 16 shows that the antero-posterior movement of the mesoscutum is greater during flapping flight at 28·6 beats per sec. than during normal flight. Also the elevation of the mesoscutum was much smaller. Expressed as percentages of total movement of the sclerite involved, within its plane of movement, these scutal ascensions are:

Mesoscutum, flapping flight	8·75 %	Mesopostnotum, full flight	15·0 %
Mesoscutum, full flight	17·2 %	Metascutum, full flight	12·7 %
Mesopostnotum, full flight	14·8 %		

They are thus seen to be quite considerable.

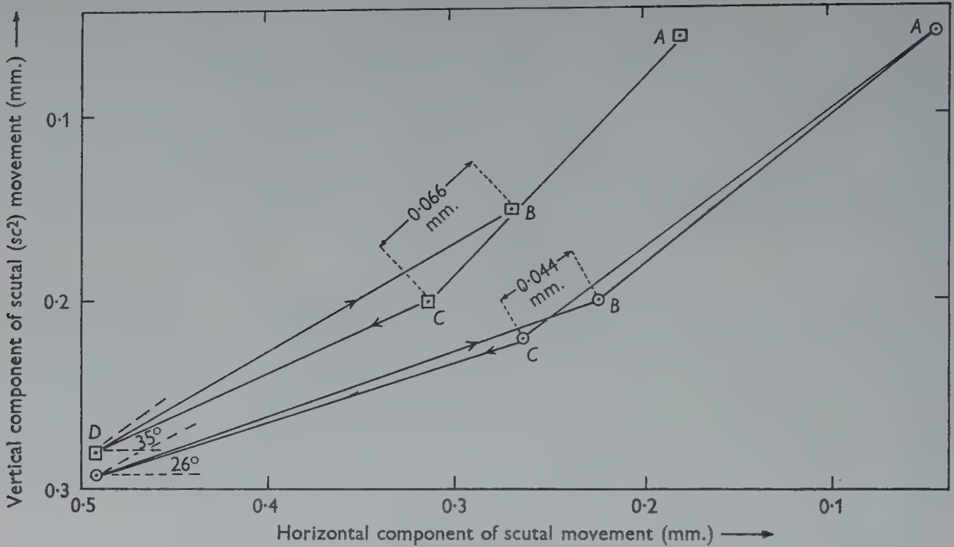

Fig. 16. Two graphs, plotted on the same scale after adjustment of co-ordinates, to show the movement of the mesoscutum during a flapping wingstroke (circles) and a full flight wingstroke (squares). Body angle 7°. Therefore true angle of movement during flapping flight, 26°+7° = 33°: and during full flight, 35°+7° = 42°. N 28·6/sec. during intermittent flapping and 35/sec. during full flight. The scutal ascension is indicated. A, Forewing full down at $\Gamma_{\text{min.}}$; B, mid-position of Γ (downstroke); C, mid-position of Γ (upstroke); D, forewing full up $\Gamma_{\text{max.}}$).

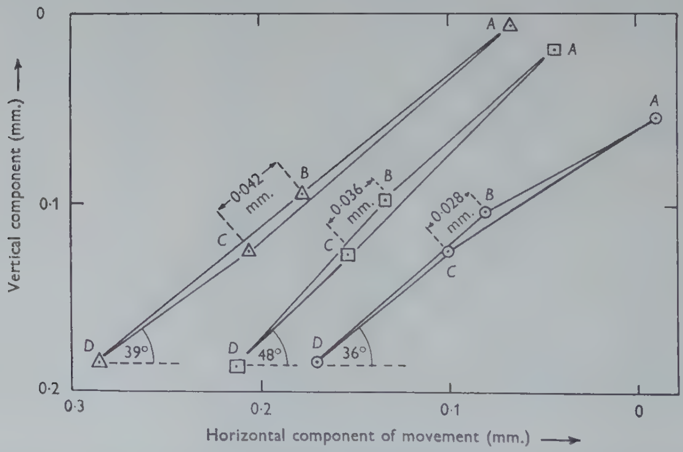

Fig. 17. Three graphs, plotted on the same scale after adjustment of co-ordinates, to show the movement of the mesopostnotum (triangles and squares) and of the metascutum (circles), all during full flight. The ascensions of both sclerites are indicated. A, B, C and D as in Fig. 16.

(*f*) *Posterior lobe of the lateroprescutum*

The movements of the posterior lobe of the lateroprescutum follow the movements of the scutum, to which it is medially fused. But the action of the anterior coxoalar muscle, which pulls the anterior lobe of the lateroprescutum down distally and posteriorly, causes slight outward bending of the posterior lobe of the latero-prescutum, to which it is fused, during the last 10° of the downstroke and during part of the upstroke (Fig. 18).

(*g*) *Prescuta and tegulae*

The horizontal component of the movement of the mesoprescutum was measured, and compared with that of the mesoscutum. The two were found to move together as a unit. The horizontal component for both from $\Gamma_{\text{max.}}$ to $\Gamma_{\text{min.}}$ was 0·36 mm.

Similarly, the metaprescutum and metascutum were found to move together as a unit, the value from $\Gamma_{\text{max.}}$ to $\Gamma_{\text{min.}}$ in this case again being 0·36 mm.

The movement of the mesotegula, a very weakly sclerotized plate lying in the membrane behind the ante-alar ridge of the mesothorax and in front of the mesoprescutum, was measured. The horizontal component from $\Gamma_{\text{max.}}$ to $\Gamma_{\text{min.}}$ was backward by 0·05 mm. The horizontal component of the metategula, which is similar in structure to the mesotegula and lies distal to the mesopostnotum in the pleural wing membrane, was backward by 0·09 mm. from $\Gamma_{\text{max.}}$ to $\Gamma_{\text{min.}}$

(*h*) *Mesopostnotum*

An attempt was made to find whether or not the mesopostnotum contributed towards the control of phasing between the two pairs of wings, and whether any large forces were transmitted from mesothorax to metathorax or vice versa. A slit was therefore cut across this sclerite, over the air sac to avoid blood loss, and the insect was then flown at a windspeed of 2·5 m./sec. and 24° C. The movements of the nota were observed stroboscopically and the results showed that:

(1) The part of the mesopostnotum posterior to the slit moved down with the metascutum during the downstroke.

(2) The part of the mesopostnotum anterior to the slit moved down with the mesoscutum.

(3) There was no alteration of the normal movements of the scuta, as far as could be observed.

Thus each segment of the pterothorax functions as a unit, and no great forces are transmitted through the mesopostnotum. The various stroke parameters measured showed no deviation from those of an unmutilated specimen: $\Gamma_{\text{max.}} = 115°$, $\Gamma_{\text{min.}} = 70°$, pleural ridge angle = 34°, stroke plane angle = 26°. The phase difference also remained constant.

(*i*) *Metapostnotum*

This sclerite bears no muscle attachments and is moved by the metascutellum, which in turn is moved by the metascutum being fused to it. Thus, during the upstroke, when the metascutum moves downwards and forwards, so also does the metascutellum, and the anterior edge of the metapostnotum therefore tilts downwards and forwards. The reverse movement occurs during the downstroke (Fig. 18*c*, *d*). The metapostnotum connects the antecosta of the first abdominal tergum, which carries the insertion of the oblique intersegmental muscle, to the metanotum.

Thus, if this muscle acts as a wing depressor, as suggested by Clark (1940), cutting through the metapostnotum should prevent the transmission of its force to the hindwings. Accordingly, the metapostnotum was cut through transversely on a male *Aeshna juncea*, and the insect flown as normal. As when the mesopostnotum was cut, the values of stroke parameters were unaffected by this slit. The oblique intersegmental muscle cannot therefore function as a flight depressor muscle; its real function will be shown in §IV(*d*).

IV. THE RELATION OF MUSCLE CONTRACTION SEQUENCE TO WING KINEMATICS

The fact that the stroke is controlled by small muscles in Odonata made it possible to use the technique of cutting these in various combinations without appreciably affecting the muscular power of the stroke. The insects used for the experiments could therefore be flown under constant conditions so that the wing parameters, exclusive of rotation, remained reasonably constant also. The only variables were then the angles of supination and pronation, and changes in these could thus be related to the elimination of various small controlling muscles from the contraction sequence. In some of the experiments main flight power muscles were also cut, and in these the stroke frequency was, as would be expected, less than during a normal wingstroke, though the phase difference between fore- and hindwings still remained the same. Preliminary experiments were qualitative, and observations made during these facilitated interpretation of later quantitative studies. The muscle transection experiments were made possible by the large extent of the pleural wing membrane in the dragonfly, enabling individual muscles or groups of muscles to be cut through the membrane. The cuts were of course restricted to a minimum size to avoid excessive damage to the pleural wing membrane.

(*a*) *Phase difference and stroke plane angles during a normal wingstroke*

It is first necessary to describe the normal wingstroke at 24° C. and 2·5 m./sec. windspeed. The hind wings move ahead of the forewings during each half-cycle of the stroke. In *A. juncea*, when the forewings were down at $\Gamma_{\text{min.}} = 70^\circ$, the hindwings were ascending at $\Gamma = 90^\circ$: $\Gamma_{\text{max.}}$ was 115°. In *A. cyanea*, with the forewings down at $\Gamma_{\text{min.}} = 70^\circ$, the hindwings were ascending at 92°. With the hindwings down, the forewings were still descending at 110°: $\Gamma_{\text{max.}}$ was 120° in this species (Fig. 18, *e*, *f*). This means that when the hindwings reach the end of the downstroke they are supinated: the forewings are at this instant pronated. Also, as the hindwings pronate at the end of the upstroke, the ascending forewing is still supinated.

In *A. juncea* the stroke plane angle was 33° from vertical. After correction for body angle of 7°, the true stroke plane angle is 26° for both fore- and hindwings. The pleural ridge angle, i.e. the angle between the pleural suture and the vertical, was 41°, which after correction for body angle is 34°. In *A. cyanea* the true stroke plane angle is 33° and the true pleural ridge angle 38°. Thus, the stroke plane angle is almost in the same plane as the pleural ridge.

(*b*) *Upstroke supination experiments*

The apodeme of the anterior coxoalar muscle was cut through just below its insertion into the anterior prong of the lateroprescutum, first in the left, and then in both forewings of a male *A. juncea*. The insect was flown at 2·5 m./sec. and 23° C. There was no change from normal values of stroke frequency or angle of wing-stroke: $N = 35$/sec.: stroke plane angle $= 26°$: $\Gamma_{\text{max.}} = 115°$: $\Gamma_{\text{min.}} = 70°$, occasionally rising to 85°.

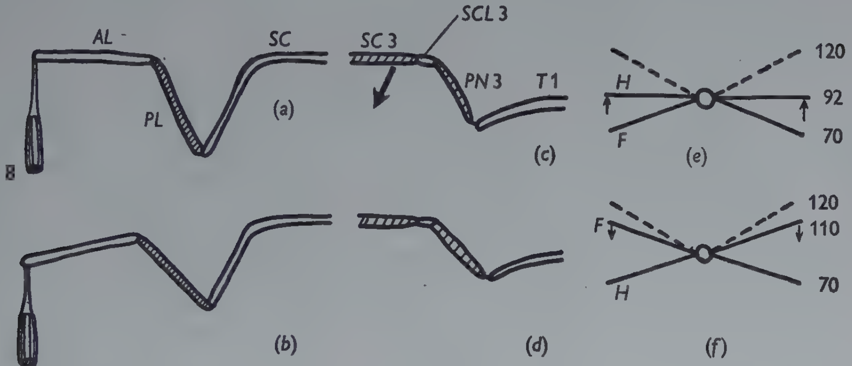

Fig. 18. (*a*) and (*b*). Diagrams to show the slight outward bending of the posterior lobe of the lateroprescutum (*PL*) with contraction of the anterior coxoalar muscle (8). (*a*) Before, and (*b*) after contraction. The diagrams are shown as transverse sections through the posterior part of the anterior lobe of the lateroprescutum (*AL*). Anterior view of right-hand section. (*c*) and (*d*). Diagrams to show the downward tilting of the anterior edge of the metapostnotum (*PN* 3) with contraction of the metathoracic elevators (indicated by an arrow); (*c*) beginning, and (*d*) end of upstroke. Drawn in longitudinal section from qualitative observations. *SC* 3, metascutum: *SCL* 3, metascutellum: *T* 1, first abdominal tergum. (*e*) and (*f*). Diagrams to illustrate the phase difference between the forewings and hindwings in *A. cyanea*. (*e*) Forewings down at $\Gamma = 70°$, hindwings already ascending at 92°: (*f*) hindwings down at $\Gamma = 70°$, forewings still ascending at 110°. All angles measured from lower vertical.

The supination twist, which was due to the contraction of the anterior coxoalar muscle at $\Gamma_{\text{min.}} + 10°$ ($= 80°$), and which was normally observed as a supination from this position and throughout part of the normal upstroke, was now cut out. But active supination still occurred at $\Gamma = 85°$ on the upstroke, i.e. much later than that coming into the stroke from the anterior coxoalar muscle (Fig. 19, *a*, *b*). It is thus concluded that another supinator muscle must be operating during the upstroke. The value of $\Gamma_{\text{min.}}$ tended to rise occasionally to 85°, so that the wing was then beating with the supination twist due to this other supinator muscle occurring at the beginning of the upstroke.

It was suspected that the third subalar muscle, which inserts on the axillary complex by an elastic tendon, could be causing this additional supination. Accordingly, the elastic ligament was cut on the same specimen, just below its insertion. The insect was flown at the same speed and supination was now found to be absent from the upstroke altogether. Cutting this muscle had thus cut out the residual supination (Fig. 19, *c*). The third subalar muscle was now cut in an otherwise intact specimen. The wingstroke showed that supination from the anterior coxoalar muscle lasted until $\Gamma = 90^\circ$ on the upstroke; supination due to the third subalar was cut out (Fig. 19, *d*).

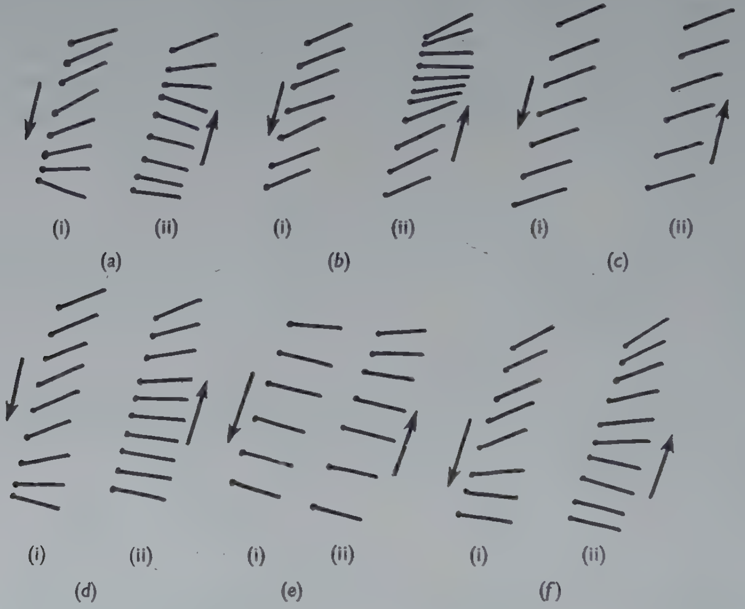

Fig. 19. Diagrams showing wingstrokes in consecutive sections under various conditions. The leading edge of the wing is shown by a dot. Series (i) are downstroke, and series (ii) upstroke. (*a*) is a normal wingstroke; (*b*) is in an insect with the anterior coxoalar muscle cut; (*c*) is in an insect with both the anterior coxoalar and the third subalar muscles cut; (*d*) is with just the third subalar muscle cut; (*e*) has the first and second basalars and the third subalar muscles cut; (*f*) has the second subalar muscle cut. All from qualitative stroboscopic observations, explained in the text.

Thus, supination on the last 10° of the downstroke is caused by the anterior coxoalar muscle and continues throughout the first part of the upstroke. It is reinforced by the action of the elastic ligament and third subalar muscle at $\Gamma = 85^\circ$ on the upstroke. Weis-Fogh (unpublished) found that in *Aeshna* the latter muscle contracts tonically during both upstroke and downstroke. Thus, this muscle, previously regarded by Clark (1940) as a downstroke depressor, acts to cause supination from $\Gamma = 85^\circ$ during the upstroke. During the downstroke its supination effect is overcome by the much greater downstroke pronation forces, but the muscle does in fact function as an antagonistic supinator/depressor. (It must function as a depressor whilst causing supination on the upstroke, but this force is small compared to the elevator muscle forces.)

When the upstroke supinators are cut, the wing remains pronated throughout the upstroke. This may be due to the action of the tergosternal elevators or to mechanical properties of the pterothoracic skeleton. Whatever the cause, it follows that the pronating forces are normally opposed and overcome by the upstroke supinators.

(*c*) *Downstroke pronation/supination balance experiments*

A series of experiments was now undertaken to demonstrate the existence of a balance of supinating and pronating forces during the normal cycle of flight movements. The large second basalar muscle was cut in a specimen with the third subalar already cut. The small first basalar was inadvertently cut with the second basalar. Since the third subalar was cut, this muscle could not be responsible for supination on the downstroke. However, the wing was supinated throughout the whole stroke, except for a slight pronation just before the end of the upstroke (Fig. 19, *e*). The downstroke supination must therefore have been produced by either the first subalar or the second subalar, or by both; the upstroke supination was again by the anterior coxoalar.

Thus the large pronation during the last part of the normal upstroke, and during the downstroke, must be due to the contraction of the first and second basalar muscles. These therefore contract before the end of the upstroke. The slight pronation just before the end of the upstroke must have been caused either by the tergosternal elevators or by mechanical forces in the pterothoracic cuticle; its presence could now be detected because of the absence of the synchronous supination force from the third subalar muscle. During this experiment the wingstroke frequency dropped to 25 per second, i.e. to the speed of the wing with approximately half of its downstroke power removed, indicating the extent to which the second basalar also acts as a flight power muscle.

The second subalar muscle was now cut through the pleural wing membrane, and the wingstroke pattern was again observed (Fig. 19, *f*). A large pronation, caused by the first and second basalars, now appeared in the wingstroke at $\Gamma_{\text{max.}}-5^\circ$, and was much more pronounced than in a normal stroke (qualitative observation), in consequence of the lack of reduction of pronation by the second subalar. Wing frequency dropped to 32·4 per sec. The first subalar muscle was now cut in the same insect, i.e. together with the second subalar, but there was no detectable difference in wingstroke from an insect with only the second subalar cut. The frequency dropped to 31 per sec. With only the first subalar cut the normal wingstroke was observed, but wing frequency again dropped to 31 per sec.

Thus whereas the smaller second subalar muscle produces a large supination leverage about the fulcrum formed from the pleural wing process, the first subalar muscle causes no detectable effect of this kind. It is therefore the second subalar muscle which, during the forewing downstroke, causes supination control of the pronation resulting from the action of the basalar depressors.

The insect was able to fly with all four first subalars cut, one in each wing. But with the second subalar muscles cut as well, overpronation rendered it incapable of stable flight, though the phase difference was unaffected. Thus the second subalar must contract with the first and second basalars preventing over-pronation and assisting them synergically as depressors in the downstroke.

Rotation angles were measured for a normal stroke, for an insect with the second subalar cut, and for one with the anterior coxoalar cut (Table 6). However, these

Table 6. *The effect of cutting various muscles on the angles of rotation*

Wing position Γ	Angle of rotation (from horizontal) Normal	Second subalar muscle cut	Anterior coxoalar muscle cut
Max. 115°	22° *p*	47° *p*	22° *p*
Min. 70°	33° *s*	33° *s*	10° *p*
85° *d*	7° *p*	10° *p*	37° *p*
105° *d*	47° *p*	57° *p*	47° *p*
90° *u*	18° *s*	18° *s*	18° *s*
105° *u*	7° *s*	7° *s*	7° *s*
80° *d*	2° *p*	2° *p*	22° *p*
95° *d*	37° *p*	40° *p*	37° *p*
85° *u*			23° *s*

Key. d, downstroke: *u*, upstroke: *p*, pronation (corrected for body angle 7°): *s*, supination (corrected for body angle 7°).

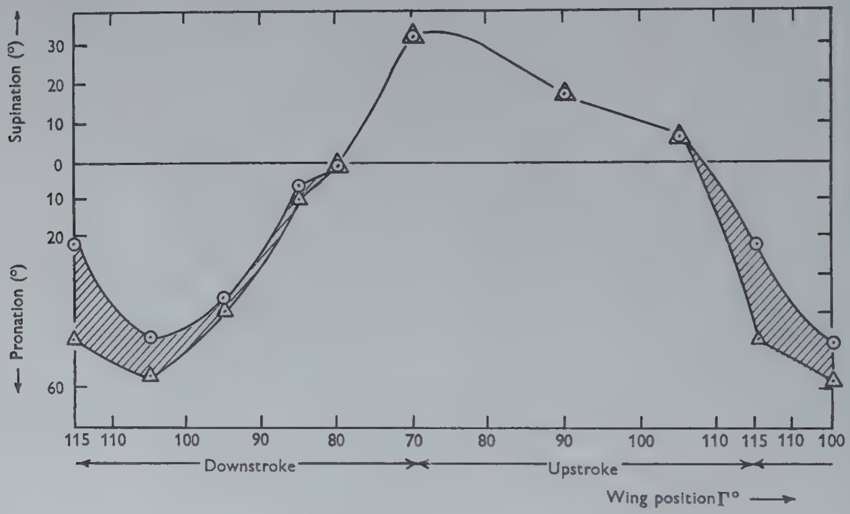

Fig. 20. Quantitative results of the effect on the wingstroke of cutting the second subalar muscle in a male *A. juncea* forewing (triangles), compared with a normal wingstroke (circles). The over pronation caused by this transection is indicated by the hatched area between the graphs.

angles were measured in plane projection on the median-longitudinal (sagittal) plane, and so require perspective correction for the stroke angle and wing position Γ. This calculation involves complex spherical transformation trigonometry and proved too difficult. When looking at the graphs of these results therefore (Figs. 20, 21), it should be borne in mind that the absolute values of rotation angle are nearly correct only for two positions, Γ = 90° on each half stroke, the other positions exaggerating the angles involved. Nevertheless, the graphs can give useful qualitative impressions of the rotation control by means of the small muscles involved.

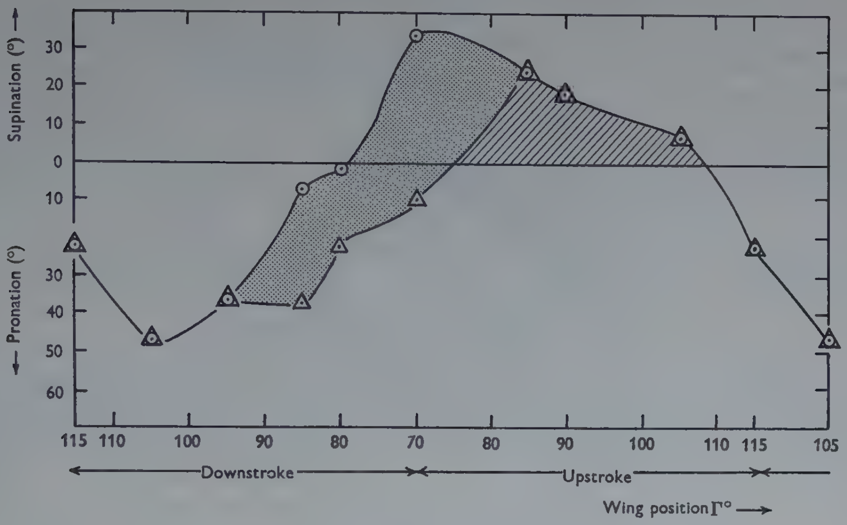

Fig. 21. Quantitative results of the effect on the wingstroke of cutting the anterior coxoalar muscle in a male *A. juncea* forewing (triangles), compared with a normal wingstroke (circles). The over pronation caused by this transection is shown dotted, and this represents the supination normally due to this muscle. The remaining supination, caused by the third subalar muscle, is indicated by the hatched area.

(*d*) *The function of the oblique intersegmental muscle*

Clark (1940) suggested that this muscle was a wing depressor, but it has already been shown that this cannot be so (§ III, *i*). It was suspected that it is actually concerned with the elevation of the abdomen. A reflex was found by which pushing the head resulted in an elevatory twitch of the whole abdomen about the thorax, presumably caused by the slow tonic contraction of the oblique intersegmental muscle. The insertion of this muscle was therefore cut through on both sides of the animal, through the pleural wing membrane in a male *A. juncea* just below the antecosta of the first abdominal segment.

The elevatory twitch reaction now disappeared. Thus, the function of this muscle is one of abdomen elevation. Manipulation downwards and forwards with a needle on the outside of the first abdominal tergum at the point of insertion, caused elevation of the abdomen about the two hinges; the dorsal hinge is between the metapostnotum and the first abdominal tergum, and the ventral hinge between the metathoracic sternum and the first abdominal sternum (Fig. 22).

The only connexion which the muscle has with flight is in abdomen elevation which removes the aerodynamic drag component due to the depressed abdomen in flight, thus allowing for an increase in speed. It can thus act to release the abdomen brake during slight increases in forward flight speed. It must also contract during hovering and reverse flight, when the whole body pitches forward with the abdomen raised about 20° above the horizontal (field observation on *Sympetrum striolatum striolatum* (Charpentier)).

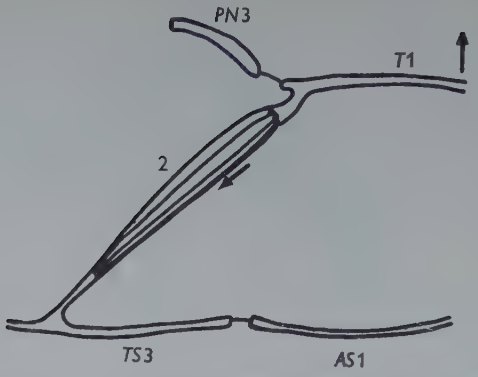

Fig. 22. Diagram to show the elevation of the abdomen by the oblique intersegmental muscle (2) of the metathorax, about the dorsal hinge between the metapostnotum (*PN* 3) and the first abdominal tergum (*T* 1), and the ventral hinge between the metathoracic sternum (*TS* 3) and the first abdominal sternum (*AS* 1).

V. DISCUSSION

(*a*) *Promotion and remotion*

The results may be discussed by giving an account first of promotion and remotion and then of the muscle balances and the functional division of the wingstroke. All aspects of the subject are then brought together to give an interpretation of the normal wingstroke.

No muscles have so far been shown specifically to cause promotion or remotion of the wings in Odonata. Clark (1940) actually states that no promotion or remotion occurs in dragonflies. But Magnan (1934), who plotted wingtip paths, showed promotion and remotion in the cycle. In fact, however, he grossly exaggerated these in relation to the rest of the stroke. As in *Schistocerca*, slight promotion can be observed stroboscopically at the end of the upstroke, and slight remotion at the end of the downstroke; the wingtip pattern of the dragonfly is therefore a thin ellipse.

The plane of the main power muscles is at an angle to the vertical, and varies from species to species. Thus when these muscles contract along their lines of action (Fig. 6, *B*), the forces produced have a large downward component (*D*) and a small forward component (*F*). Considering first a depressor muscle, e.g. the first basalar or first subalar, the component *D* causes wing depression about the pleural fulcrum. The small component *F* causes slight promotion of the wing. This, however, is a sort of passive promotion; as the wingstroke plane is at an angle to the vertical, the wing is automatically more forward at the end of the downstroke than at the beginning. But if, as seems likely, the depressors begin to contract before the end of the upstroke, as they do in *Schistocerca*, then the small forward component *F* is also able to cause an active promotion when the momentum of the wing is smallest—i.e. at the top of the stroke. In other words, active promotion by the depressor muscles precedes their depressor action.

Similarly, considering upstroke muscles, these pull in the same line and their components have the same relative values as the depressor muscles, but the forces are of course translated by different relationships of load, effort and fulcrum. The large downward component, D, is now translated into an elevation force through the wing hinges. The small forward component, F, is transformed into a remotion force through the forward movement of the notum and consequent backward movement of the wings. (It is important to note that the nota move in approximately the same line as the wings, but in exactly the opposite sense; when the nota move up and back, the wings move forward and down and vice versa.) Just as with promotion, remotion occurs passively throughout the upstroke, but assuming as before that the elevator muscles begin to contract before the end of the downstroke, then active remotion will occur when the wing momentum is lowest, i.e. at the bottom of the wingstroke.

Remotion at the bottom of the downstroke is also assisted by the action of the anterior coxoalar muscle which causes movements 1 and 2 (Fig. 11) between the humeral and axillary complexes. This results in remotion about the line of weakness between the two plates (Fig. 10). It is important to note that during a normal wingstroke the movements are the opposite of those which would be expected from observation of a manipulation stroke alone (Figs. 13, 14). The anterior coxoalar muscle is thus very specialized, functioning in elevation and supination and in assisting remotion.

In these ways, therefore, promotion and remotion are correctly phased into the wingstroke.

Before leaving the question, however, one should also note that the absence of a wing-flexing mechanism in the Odonata eliminates one problem concerning promotion in the wing-flexing Pterygotes—i.e. that of the forces required to promote the wings from the flexed position, and to hold them out during flight. No doubt the absence of the wing-flexing mechanism, which is correlated with the fusion of axillary sclerites in the Odonata, has contributed towards their very efficient flight and the subsequent reduction of their walking powers (Neville, 1960).

(*b*) *The muscle balances and functional division of the wingstroke*

In the following discussion of the relation between muscle contractions and wing kinematics, the wingstroke is considered in four phases:

(i) Upstroke from $\Gamma_{\text{min.}}$ to $\Gamma_{\text{max.}}-5^\circ$.
(ii) Upstroke from $\Gamma_{\text{max.}}-5^\circ$ to $\Gamma_{\text{max.}}$
(iii) Downstroke from $\Gamma_{\text{max.}}$ to $\Gamma_{\text{min.}}+10^\circ$.
(iv) Downstroke from $\Gamma_{\text{min.}}+10^\circ$ to $\Gamma_{\text{min.}}$

During phases (i) and (iv) the first tergosternal and the anterior coxoalar act synergically as elevators, but as synchronous antagonists in pronation/supination control. It may be worth noting here that the instantaneous rotation position of a wing is determined by the relative extents of contraction of the opposing muscles causing pronation and supination about the pleural fulcrum. These extents of contraction could be caused either by the fraction of muscle fibres involved, the number of units in the muscle, or by a differential timing sequence of contraction. The first tergosternal and the third subalar both act as antagonists with respect to elevation/depression and pronation/supination. The third subalar and the anterior coxoalar act antagonistically during depression/elevation but synergically in supination. The third subalar is thus an antagonist to all three elevators: first tergosternal, anterior coxoalar and posterior coxoalar.

During phases (ii) and (iii) the second basalar and the second subalar act synergically to give depression but antagonistically to control pronation/supination. Similarly the second basalar and the third subalar act synergically in depression but antagonistically in pronation/supination. The second and third subalars both act synergically with respect to both depression and supination.

It can be seen from these results that although visually the wingstroke divides into downstroke and upstroke at $\Gamma_{\text{max.}}$ and $\Gamma_{\text{min.}}$, functionally it divides at $\Gamma_{\text{max.}}-5^\circ$ upstroke and $\Gamma_{\text{min.}}+10^\circ$ downstroke. So the functional upstroke is between $\Gamma_{\text{min.}}+10^\circ$ (on the visual downstroke) and $\Gamma_{\text{max.}}-5^\circ$ (on the visual upstroke); the functional downstroke occupies the rest of the stroke between these two points.

(*c*) *The typical wingstroke*

The wingstroke cycle can now be described, stage by stage, starting with the supinated wing 5° from its highest position on the upstroke.

The two basalar depressors and the first and second subalar depressors (all phasic muscles) all presumably contract at this point, together with the tonic third subalar muscle which exerts a steady tension throughout the stroke. The decelerating wing then reaches its highest point and becomes pronated by dominance of the basalars in the basalar/subalar rotation balance. The fact that the large first subalar has almost no supination leverage inevitably results in the wing descending pronated by the large second basalar muscle. All the phasic downstroke muscles presumably begin contracting isometrically, being restrained from shortening by the ascending wing. They will thus develop maximum tensile force by the beginning of the visual downstroke. At this stage of the cycle the nota reach their lowest and most anterior position, having been pulled down by the tergosternals and coxoalars. Each notum moves as a unit consisting of prescutum, scutum and scutellum with their corresponding lateral regions, the lateroprescutum, lateroscutum and axillary cord. Promotion occurs at this point in the cycle as discussed above.

While the pronated wing is descending the nota are levered upwards by the wing about the wing-base hinges. Another factor probably contributing to their movement is the release of elastic energy stored in the elastic ligaments of the wing-base. Weis-Fogh (unpublished) has calculated this force for *Schistocerca* and shown that it is the factor which gives rise to notal ascension; during the downstroke the nota are pushed upwards by the stored energy and so occupy higher positions than they do for the corresponding position of the wing on the upstroke. The same explanation is probably valid for dragonflies.

When the descending wing reaches a point 10° from the bottom of the stroke the anterior coxoalar elevator contracts, causing the lateral margin of the anterior prong of the lateroprescutum to move suddenly downwards. This force is transmitted across to the posterior edge of the humeral complex which suddenly snaps away from the axillary complex about a line of weakness between them. A complex propeller-like supination section then develops out along the wing. The shape of this section is controlled by the venational pattern, and will be dealt with elsewhere. It is probable that the first tergosternal and posterior coxoalar elevators also begin to contract with the anterior coxoalar at this point. Like the depressors, their contraction is probably isometric until the wing reaches its lowest point. The sudden snap action of the movements between the humeral and axillary complexes is a good indication that the anterior coxoalar muscle contracts more strongly initially than during the remainder of its contraction period, probably caused by stretching of the series elastic component in the muscle by the momentum of the wing. Remotion then occurs as discussed above.

The wing remains supinated throughout the functional upstroke (i.e. to $\Gamma_{\text{max.}} - 5^\circ$). This supination is effected during the first half of the upstroke by the phasic anterior coxoalar muscle, and during the second half by the tonic third subalar.

There is no mechanical control of phasing from metathorax to mesothorax via the intervening mesopostnotum. This, together with the fact that there was no change in wing phasing during the muscle transection experiments, seems to indicate that phasing is under neural rather than mechanical control.

VI. SUMMARY

1. Movements of the wings and pterothorax of *Aeshna* spp. have been studied by stroboscopic observation combined with the cutting of certain muscles.

2. There is a constant phase difference between the contractions of the mesothoracic and metathoracic flight muscles, and a resulting phase difference between the movements of the wing-base sclerites and wings in the two pterothoracic segments.

3. As the wings in each segment move upwards and backwards, the respective notal sclerites are moving forwards and downwards. Conversely, as the wings beat forwards and downwards, the nota move backwards and upwards.

4. The anterior coxoalar elevator muscle pulls on the anterior lobe of the lateroprescutum and so, via the membrane on the posterior edge of the humeral complex. This causes extensive supination of the wing, important in hovering and backward flight, and is due to a sudden snap-like separation of the humeral and axillary complexes about a line of weakness between them when the descending wing is at 10° from its lowest position.

5. Both remotion at the end of the downstroke, and the complex propeller-like supinated wing section which develops during the last 10° of the downstroke and most of the upstroke, are made possible by the line of weakness running along the membrane between the two complexes.

6. Comparison of manipulated wingstrokes with normal wingstrokes showed that supination is actively controlled in the flying insect by muscular contraction.

7. Scutal ascension, described in the paper, was detected. At any point on the downstroke, the scuta occupy higher and more posterior positions than they do at the corresponding point on the upstroke.

8. Fore and aft movements of the nota are greater during flapping flight at low wingbeat frequency, than during full flight at higher wingbeat frequency.

9. The prescutum, scutum and scutellum move together as a unit up and down and fore and aft, throughout the wingstroke cycle.

10. Supination during the last 10° of the downstroke and the first half of the upstroke is due to the anterior coxoalar muscle, and during the second half of the upstroke to the third subalar muscle. Downstroke pronation/supination balance is controlled by the basalars and second subalar. A detailed summary of these rotation balances is given in the discussion.

11. The oblique intersegmental muscle of the metathorax is shown to be an abdomen elevator and not a wing depressor. The functions of the various flight muscles are demonstrated experimentally for the first time. The anterior coxoalar muscle is shown to be specialized, functioning in elevation and supination and assisting remotion.

I wish to express my sincere thanks to Prof. Torkel Weis-Fogh for his help and hospitality, and for making some of his unpublished material available, throughout my three-month stay in his laboratory in Copenhagen, during which this work was done. My thanks are also due to the Department of Scientific and Industrial Research for financial assistance; and to Prof. O. W. Richards and Mr R. G. Davies, Department of Zoology and Applied Entomology, Imperial College, London, for helpful discussions.

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